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RESEARCH INTO THE FACTORS EFFECTING THE BIOCONVERSION OF CATTLE MANURE AND CELLULOSIC MATERIAL MIXTURES TO BIO-GAS

Prepared for
MONTANA DEPARTMENT of NATURAL RESOURCES and CONSERVATION

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RESEARCH INTO THE FACTORS EFFECTING THE BIOCONVERSION OF CATTLE MANURE AND CELLULOSIC MATERIAL MIXTURES TO BIO-GAS

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I. Objectives

In the proposal submitted I laid out two broad objectives and four specific objectives for this study. The broad objectives were (1) to inspect the possibilities of multiple products from anaerobic fermentation of wastes (viz. fuel, feed and fertilizer) and (2) to check the possibilities of increasing the efficiency of fuel production by this process.

The four specific objectives are enumerated below:

1. Ascertain the optimum carbon/nitrogen ratio for maintenance of good bacterial growth.
2. Monitor methane production potential as additional carbohydrate (cellulose) was made available in waste.
3. Check the possibility of removing ammonia from fermenter digest.
4. Perform analyses on nitrogen content, pH, ammonia, and fermentable carbohydrate in feedstock and residue.

An assessment of these objectives has been useful for predicting the economic feasibility of fuel production by anaerobic fermentation. Further, this study has led to elucidating some specific parameters which need investigation. It is clear from the results, thus far, that a certain plant material (viz. lignin) poses a problem to efficient conversion of the fermentable carbohydrate. Although this was not an objective it is a very important discovery.

II. Experimental Approach

The waste materials used in this study were agricultural wastes; cattle manure and wheat straw. These wastes are produced in large amounts in the state. The study has broader implications, however, since a similar study could be applied to municipal wastes, forest product wastes, other animal manures and other cereal straws.

This study uses the endogenous microbes (natural occurring bacteria) of the cattle fecal matter. We allowed cattle manure to obtain anaerobic conditions in a glass fermenter by simply adding manure to the container and sealing it off from surrounding atmosphere. Gases produced from the fermentation were vented through a gas meter and the effluent gases could be collected and analyzed

for methane and carbon dioxide. The feeding was accomplished by a batch process, i.e. a certain quantity would be added and removed daily. The feed stock manure was premixed to a specific content of solids, packaged and frozen. This permitted a consistent diet throughout a given experiment.

Chemical analyses were performed on feed stock and digest which was removed from the fermenter. The major analyses performed were pH, total oxidizable carbon, dry weight of solids and ammonium ion concentration. Fewer analyses were performed to monitor volatile fatty acids and glucose. Glucose content was checked several times and found to be virtually zero, thus additional analyses were not performed.

The inital fermenter was set up in such a way as to provide data concerning fuel production from untreated manure in a "pit type" fermenter. This would provide a baseline for comparison to treated manure, process changes, additions or fermenter design. This was essentially just raw manure from which any large pieces of debris had been removed. The manure was mixed with enough water to provide approximately 8% solids. The mixture was not agitated or stirred. Feed (manure) was added (400 ml) and digest (400 ml) was removed daily. The total volume was approximately 8 liters. The temperature was maintained at 37°C. The pH obtained was 7.1 to 7.2 and the average gas volume produced was 0.02 cubic feet per liter of ferment per day. This fermentation was allowed to run for 36 days.

The first variable that was changed was to blend the manure and water when mixing up feed stock. This reduced the particle size considerably and provided a more manageable viscosity. The solid content was maintained at ca. 8%. All other parameters were held constant. The average gas volume produced was increased to a value of 0.033 cubic feet per liter per day. This experiment was run twenty days. The increase in gas produced suggested that grinding or blending the waste material would significantly increase gas production.

These initial experiments allowed us a chance to evaluate the fermenter design and establish a protocol for running experiments. Since we had only one fermenter at this time it seemed appropriate for each variable introduced that a baseline must be established first, manure content should be consistent and temperature and pH should be maintained constant throughout the experiment.

Further, we noticed that if the fermenter was agitated more gas could b released. This suggested that some means of stirring the ferment should be introduced. This would require fabricating a new fermenter. Since there were many variables we wanted to observe, we also felt it necessary to have several fermenters operating simultaneously. In experiments with a single fermenter one should operate on one stock pile of waste feed stock for 15 to 20 days to establish the baseline and then change some parameter and ferment for an additional 15 to 20 days and compare the averaged results to assess the impact of the change. This was the mode of operation for the nex few experiments until we obtained new fermenters.

As mentioned previously pH, % solid of digest and feed, total oxidizable carbon of feed and digest, ammonium ion concentration and the ratio of methan (CH_4) and carbon dioxide (CO_2) were monitored. Since some of these were assayed daily it would require many pages to report the data here. Thus we have reported averaged values for given experiments.

Before going on with additional experiments, we would like to describe the biochemical and microbial activity which occurs in anaerobic fermentation This is necessary to present a logical discussion of the potential to increas the efficiency of fuel production.

First, there is a delineation of microbial activities. One type of activity concerns degradation of biopolymers to small and/or simple molecules These simple molecules constitute the basic energy source for the participati bacteria. These simple molecules are degraded further to even smaller molecules which represent their waste. These latter molecules serve as energy fo bacteria capable of producing methane, CH_4. This process can be represented by biochemical formulations and is best represented by carbohydrate which is in greatest quantity in waste materials. Thus cellulose, a polymer of the simple sugar, glucose, would be processed as shown by the following general scheme:

1. cellulose + H_2O ⟶ (n) glucose
2. glucose ⟶ 2 Acetic acid + $2CO_2$ + $4H_2$
3. 2 Acetic acid ⟶ 2 CH_4 + $2CO_2$
4. CO_2 + $4H_2$ ⟶ CH_4 + $2H_2O$

The first and second reactions are handled by the so called "acid formers"; several species of anaerobic bacteria. The third and fourth reactions are performed by the methane producing bacteria, methanogens. With this general scheme in mind one can postulate either a chemical process that may be rate limiting or a population of anaerobic bacteria may be rate limiting. It is necessary to identify the rate limiting process (i.e. the slowest process) in this sequence in order to address the question of how to improve the efficiency of anaerobic fermentation. If this step can be identified then studies can be initiated which would lead to faster and more complete breakdown of waste material. In other words, the fuel production from a given amount of waste would be increased in a defined period of time.

Our initial thoughts were, that the amount of carbohydrate available for conversion to methane might be a limiting factor. The waste material contains "free" cellulose and cellulose in complex with other plant polymers such as, hemicellulose and lignin. This association of cellulose with lignin could greatly reduce the amount of glucose produced from cellulose (reaction 1) and subsequently limit the amount of methane evolved (reactions 3 & 4). We chose to study the possibilities of improving the cellulose conversion to glucose by trying to breakdown the lignin in the waste plant material. It is important, however, that each step be evaluated in the overall sequence. Furthermore, it is possible microbial populations may be artifically adjusted such that an enrichment of a more efficient strain of bacteria would result in greater fuel production.

The foregoing discussion and the brief description of preliminary experiments is offered to explain or illustrate our logistics in approaching the problem of increasing the efficiency of anaerobic fermentation of wastes. There are some possible benefits other than increased fuel production. One important feature would be the increased bacterial growth that may result would enrich the residue from the process. It would be enriched in nutritive value for livestock feed. The use of the residue as feed has been tested here by Dr. James Boyd. In rat feeding trials he found that he could reduce normal feed rations by 25%. These kinds of feeding experiments are evident now from many sources. Not many are well documented. The liquid residue will contain nitrogen mostly in the form of ammonium salts which can serve as fertilizer.

III. Experimental Results

A. Preliminary Evaluation of Rate Limiting Process.

The initial experiments involved evaluating the above reactions, 1,2, and 3. Rate limiting in our interpretation here means: is the normal fermentation of manure limited by one of these reaction rates: This was tested by seeding the manure with chemical compounds shown as reactants, that is, cellulose, glucose or acetate. If any of these reactions was already operating at their maximum potential, then no increase in products would be observed when the chemicals were added. The fermentations were performed on manure; 8% solid, and manure, 8% solid, plus 1% by weight cellulose or glucose and 2% by weight glucose. This amounts to four fermentation experiments which were run a minimum of 22 days each (some as long as 36 days) with 6-8 days between experiments as an adjustment period. The total time consumed was 6 months. Some delays were experienced due to equipment failure (faulty gas meter). Table I shows the results of these experiments.

Some practical conclusions can be drawn from the data summarized in Table I. One, additional carbohydrate permits increased methane production, two, there is a slight shift in gas composition, less methane and more carbon dioxide and three, the conversion of solids to gas remains fairly constant; 34% to 37% conversion. Additionally, the experiments show that reactions 1 and 2 are not rate limiting in the fermentation under these conditions.

The increase in gas production can be calculated as follows:

methane produced per liter of digest: = gas volume produced x methane fraction.

$ft.^3\ CH_4 = 0.027\ ft.^3 \times 0.6 = 0.016\ ft.^3$

$ft.^3\ CH_4 = 0.036\ ft.^3 \times 0.58 = 0.021\ ft.^3$ 1% cellulose

$ft.^3\ CH_4 = 0.038\ ft.^3 \times 0.58 = 0.022\ ft.^3$ 1% [illegible]

$ft.^3\ CH_4 = 0.051\ ft.^3 \times 0.55 = 0.028\ ft.^3$ 2% cellu[illegible]

Thus, 1% additional carbohydrate produced a 31% increase in methane and 2% additional carbohydrate produced a 75% increase in methane. The potential for producing methane by anaerobic fermentation is enhanced if additional fermentable carbohydrate is available and is also enhanced by reducing particle size as shown by the early experiments. It was shown that blending alone produced a fifty percent increase in gas production. If one can extrapolate; the 31% increase produced by the addition of 1% of cellulose would amount to a total of 95% increase or with 2% additional carbohydrate, plus blending would produce a 162% increase or more than double the production.

It seems to us that it is imperative to explore lignin removal techniques. If lignin removal can be done at a low cost then plant waste materials could be used to provide the additional cellulose.

B. The Effects of Stirring the Ferment.

At this point we had designed a fermenter which incorporated a pump. This allowed the ferment to be stirred. In order to provide a reasonable viscosity the percent solids had to be reduced to 5% from 8%. This should be kept in mind because less solid will produce less gas. The unmixed 8% feed stock when fermented produced on the average 0.033 cubic feet of gas per liter of digest in the fermenter per day. The average value from 5% feed would be expected to be correspondingly less, viz. 0.021, or 0.033 x 5/8 = 0.021 cu. ft. per liter-day. However, the experimentally determined value for mixed 5% solid feed was 0.029 cu. ft. per liter-day.

Accordingly, it would be deemed advisable to incorporate mixing capabilities into a fermenter design. The data here show that when stirred thoroughly once a day a significant increase in gas production was obtained; 0.021 cu. ft. up to 0.029 cu. ft. per unit volume per day.

C. Evaluation of Fermenter Performance with Addition of Acetate Salt versus Glucose

Reactions 2,3 and 4 of the general scheme for carbohydrate metabolism are reiterated below:

TABLE I Fermenter Performance with Additional Carbon Source

Substrate Addition	Gas Volume cu ft./l-day	pH	% Solids Feed	% Solids Digest	% Oxidizable Carbon F/D	% wt/v NH_3	$\%CH_4$ ($\pm$1%)
none	0.027	7.2	8.1	5.3	82/78	0.1	60
1% cellulose	0.036	7.2	9.2	6.0	83/78	.08	58
1% glucose	0.038	7.2	9.1	6.0	83/78	.07	57.5
2% glucose	0.051	7.2	10.2	6.4	83.5/78	.065	55

2. glucose ⟶ 2 acetate + $2CO_2$+$4H_2$

3. 2 acetate ⟶ $2CH_4$ + $2CO_2$

4. CO_2 + $4H_2$ ⟶ CH_4 + $2H_2O$

Net: glucose ⟶ $3CH_4$ + $3CO_2$

The net reaction indicates that either glucose or acetate should produce gas of equal quantities of methane and carbon dioxide. The following experiments were designed to check the possibility of acetate conversion being rate limiting and also to validate the general scheme proposed.

Again, if acetate conversion were rate limiting, then additional acetate added to the ferment would not cause an increase in gas production. This experimental objective is simple, straight forward and easily tested. The second objective is perhaps more subtle and of more academic interest. Either reaction 3 alone (acetate conversion) or carbohydrate metabolism, reactions 2,3 and 4, should produce equal amounts of CH_4 and CO_2. Since this is not observed (note 60% CH_4, 40% CO_2) there must be an additional source of H_2, perhaps from fatty acids. This would explain the lower amount of CO_2 in the effluent gases. If the amount or ratio of carbohydrate were increased, then the gas mixture should shift toward a 50/50 mixture. If acetate were added the shift toward 50/50 would also be noted.

At this time we had fabricated two new fermenters which had the capability of mixing. One fermenter was operated on 5% solids manure mix to provide baseline data. The second fermenter was operated on the same basic manure mix with the additional carbon source added, i.e. glucose or acetate. This required two separate feeding trials, + glucose vs. straight manure and + acetate vs. straight manure. The level of additional carbon source was 1% by weight. There were several interesting phenomena observed during the course of these experiments. The overall fermenter performance is shown in Table II.

It is clear from the data shown that acetate conversion is not rate limiting and that once again, glucose confirms the results obtained earlier, glucose conversion is not rate limiting under these conditions. The daily production of methane can be calculated as before:

TABLE II. Fermenter Performance with Glucose or Acetate Additions

Additions	Gas Vol H^3/l-day	pH	%Solids		%Oxidizable Carbon/dry wt.		% wt/vol NH_3		$\%CH_4$
			Feed	Digest	Feed	Digest	Feed	Digest	
None	0.031	7.37	5.0	3.1	83.5	77.5	.07	1.09	62
1% Glucose	0.042	7.45	6.0	3.2	91	80	.11	.068	58
1% Acetate	0.034	7.67	6.0	3.4	95	80	.08	.07	69

Manure only: 0.031 x 0.62 = 0.0192 cu.ft./liter

Glucose added: 0.042 x 0.58 = 0.0244 cu.ft./liter

Acetate added: 0.034 x 0.69 = 0.0235 cu.ft./liter

It was curious to see the amount of methane produced in the acetate trial was essentially equivalent to the amount produced in the glucose trial. The total gas produced was lower. We also noticed a steady increase in pH through out the acetate trial. It climbed from 7.4 to 7.7 or a difference of 0.3 pH unit. This suggested that in that experiment CO_2 was dissolving and not evolving. A few simple manometric measurements of CO_2 evolved upon acidification of fermenter digest showed that indeed the acetate trial was analogous to the glucose trial in total gas production. In the acetate trial magnesium acetate salt was used. We believe that the magnesium enhanced the increase in pH which in turn increases the solubility of CO_2. The actual ratio of CO_2 to CH_4 in the acetate trial was 44% CO_2 and 56% CH_4 once corrected for the dissolved CO_2. This supports the hypothesis that metabolism of fatty acids or noncarbohydrate material provides a gas ratio favoring CH_4.

The general scheme offered for the metabolism of glucose and/or acetate provides for equal amounts of CH_4 and CO_2. We stated that according to this scheme carbohydrate addition to the manure fermentation should shift the CH_4/CO_2 ratio closer to 1:1 and that acetate should move the ratio closer to 1:1. This is born out by the data from this experiment. The ratios of CH_4/CO_2 were as follows 1.63:1 for manure alone; 1.38:1 for glucose added and 1.27:1 for acetate added fermentations. → These data suggest that some source of H_2 other than carbohydrate metabolism is available.

A practical point was illustrated in this experiment. The variation in pH can effect the total evolution and composition of gas. An operator of a fermenter should be aware of these effects caused by a few tenths change in pH.

Another point of interest that can be extracted from these data is the comparison of the ammonium ion concentration in the feed and digest for these experiments. One objective of this study was to observe the impact of varying the carbon/nitrogen ratio. The addition of glucose or acetate increases the ratio considerably. If the nitrogen content were limiting, then no increase in bacterial population could occur. This is important if one hopes to improve the nutritional value of the residue. We interpret the data to suggest that enrich-

ment of bacterial populations does occur when additional energy is made available.

Ammonium ion serves as one of the principal sources of nitrogen for bacteria. Ammonium ion has been shown to be used for the production of amino acids, subsequently proteins, and for the synthesis of purines and pyrimidines, subsequently nucleic acids. These are essential for bacterial growth and reproduction. Thus, if the nitrogen content were limiting the ratio of ammonium ion concentration in feed and digest should remain constant and independent of any additional source of energy. The observed fact is that the ratio of ammonium ion in feed vs digest changes. When additional energy is added the amount of ammonium ion decreases in the digest. This indicates the production of new cells; an increased demand for ammonium ion.

Another observation we feel is significant is the gradual increase in activity and the gradual decrease in activity upon addition or removal of glucose (or acetate) respectively (see Fig. 1). This can be explained on the basis of increasing and decreasing bacterial population. This is most likely in light of the fact that the enzymes involved in the metabolism of these substrates are probably constitutive (constantly produced as opposed to being induced into production by the substrate). One can also argue that the curve shown in Figure 1 is not a simple reflection of substrate accumulation since the concentration remained virtually zero on a daily basis.

We would like to point out again that in this experiment one obtains similar destruction of the manure waste solids when glucose or acetate is present as when they are not. Thus the metabolite activity does not simply switch over to the easily available energy source. This is significant because it is desirable to maintain the ability of the process to reduce the total waste mass.

D. Quantitative Estimate of Acetate Utilization

One concern of this study was the efficiency of methanogenic bacteria with respect to the cellulolytic bacteria. Arguments can be advanced that would indicate that the most probable rate limiting process would reside with the methanogen's ability to metabolize acetate. We set up an experiment which allowed us to monitor the acetate utilization from the

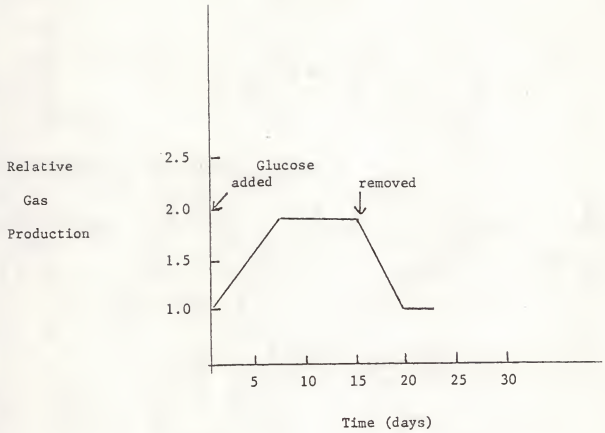

FIGURE 1 "Bacterial Enrichment" per Glucose Addition

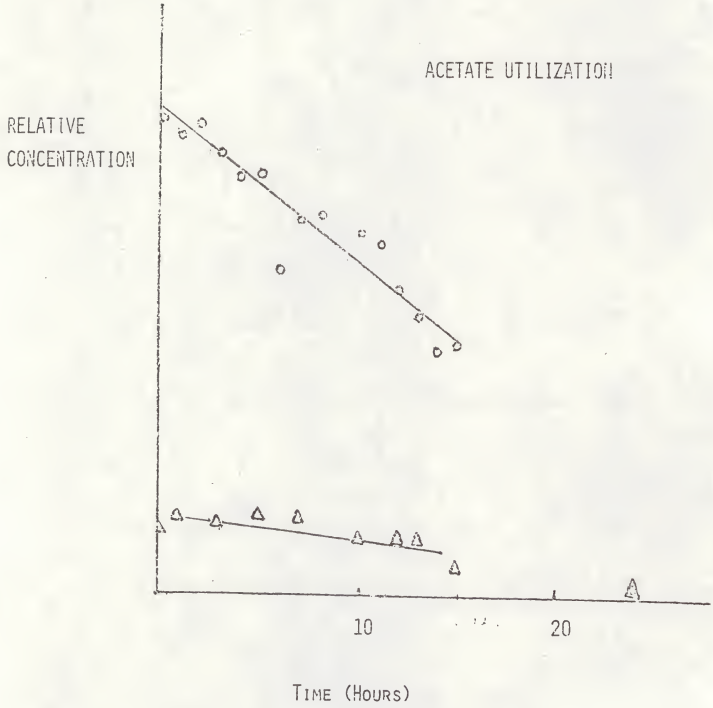
ACETATE UTILIZATION
RELATIVE
CONCENTRATION
10
20
TIME (HOURS)

TABLE III. Fermenter Performance on Added Delignified Straw

Feed	Gas Volume Cu Ft/liter	pH	% Solids		% Dry Weight oxidizable		% Wt./vol NH_3		% CH_4
			Feed	Digest	Feed	Digest	Feed	Digest	
Manure	0.028	7.5	5.4	4.0	85	78	0.09	0.115	60.5
+1% straw	0.032	7.48	6.4	4.4	82	77	0.09	0.10	59.8
+1% treated straw	0.049	7.48	6.4	4.1	84	78	0.09	0.065	57.5

time of feeding up to twenty-four hours after. These experiments were run on manure fed and manure plus acetate fed cultures. Samples were withdrawn and analyzed for acetate content every hour up to 15 hours on the acetate fed culture and every two hours on the control culture up to 15 hours and a final sample from each was taken at 25 hours. The results of these tests are shown in Figure 2. From the graph it is seen that acetate is utilized at a much faster rate when it is added in with the manure. This shows that acetate levels of approximately 0.05% are not rate limiting.

E. Fermenter Performance on Delignified Wheat Straw as an Addition to Manure.

We asked for an extension of our grant period so that we could finish this experiment. We felt that the use of wheat straw as a source of additional carbohydrate would be an appropriate test of our hypothesis concerning lignin-cellulose complex. Further, for public view this experiment would seem more appropriate although scientifically no more valid than using purified cellulose.

Wheat straw was delignified by treating with sodium chlorite. This is an oxidative process which is chemically similar to the pulping process used in paper manufacture. The process removes the lignin by making it water soluble, leaving behind the hemicellulose and cellulose fractions. These latter fractions we presume to be totally fermentable by the anaerobic bacteria present in the manure fermenters.

At this time we had fabricated a third fermenter. The experiment was set up to compare the fermentation of manure (5% solid), 5% manure plus 1% wheat straw and 5% manure plus 1% delignified wheat straw. The three fermenters allowed these fermentations to be carried out simultaneously. These experiments ran approximately 40 days. The performance of the fermenters using these three feed stocks is displayed in Table III. The figures given are averaged values.

The data in Table III reflect essentially the same performance as when glucose or cellulose were fed in addition to the manure. These data suggest waste plant material such as wheat straw can be used to supply metabolic energy for the bacteria and that significant additional methane can be produced by adding delignified wheat straw. The treatment used to remove the lignin uses a chlorite salt which is toxic to most bacteria.

The treated straw was washed subsequent to the liguin removal. The washing procedure was apparently adequate to remove any toxic residue as one can see from the excellent performance of that fermenter.

F. Experiments Involving the Removal of Ammonia from Fermenter Digest

The ammonium ion content of the fermenter digest was removed by adding a base such as lime or sodium hydroxide to raise the pH to 9.6 or 10. Air was then passed through the digest. The air carried ammonia gas with it. The amount of ammonia removed approach 100% (ca. 98%). The ammonia removed in this manner was trapped by passing the ammonia containing air through an acid trap. The acid used was phosphoric acid. The amount of ammonia trapped by this method was 95% of that removed.

The figures above suggest efficient removal of ammonia can be accomplished but upon analysis it does not seem feasible to try to obtain fertilizer by this method. The cost of equipment and chemicals along with the small amount of ammonia in the digest are not favorable. The liquid which can be removed from the digest could be easily used as is. [This liquid should be analyzed for its potential as a fertilizer in comparison with commercially available products.) Will this be done?

IV. Conclusions and Recommendations

Several conclusions can be drawn from the afore going discussion of experimental results. The fermenter trials have shown that serious consideration should be given to grinding waste materials prior to fermentation. Stirring capabilities should be included. Usually pumps are used for this purpose. These factors have been shown to greatly enhance the production of methane from anaerobic fermentation. At this point then an economic assessment should be considered for methane produced from a process with all these capabilities. The study showed that these three factors taken together provide approximately a five-fold increase in gas production. The upper limit of improving the gas production by adding in delignified plant material has not been assessed as yet but this is in progress now in our laboratory.

The optimum temperature that has been established in other studies is 98°F (37°C). We recommend insulated tanks which be located below ground level. Perhaps some assisted heating from solar heating devices could also be explored.

More research is needed on the delignification process. It seems to us that a biodegradation of lignin might alleviate some of the economic and pollutant burdens of a chemical process, such as used in this study. We have proposed and are conducting such a study now in our laboratory.

We also feel that a hard, quantitative look at the fertilizer value of the digester filtrate should be taken. This will also help evaluate the economics of the process. At present all indicators are in favor of the value of the effluent as fertilizer. These statements mostly are based on observed effects on plants. Some quantitative chemical data are needed to make a comparison with commercial fertilizers that are on the market.

APPENDIX A - METHODS

Ammonia Assay

The method is based on the reaction catalyzed by the enzyme, glutamate dehydrogenase (GDH). The reaction is shown below:

$NH_4^+ + \alpha\text{-ketoglutarate} + NADH \xrightarrow{GDH}$ glutamate + NAD + H_2O

This provides for a stoichiometric ratio between NH_4^+ and the oxidized dinucleotide, NAD. Thus, a simple spectrophotometric determination. All chemicals were obtained from Calbiochem and used without further purification. NH_4Cl from J. T. Baker was used to make standard solutions of known NH_4^+ concentration.

Preparations of fecal samples prior to assay consisted of high speed contrifugation (39,000 x g for 30 min) at a twofold dilution with 0.05 M, pH 9.8 potassium phosphate buffer. Upon completion of centrifugation, a second dilution of tenfold was performed using the same buffer solution to bring the sample to a final dilution of twenty-fold.

The dehydrogenase-ammonia assay mixture was prepared in a 1-cm path quartz cuvette of 3.3 ml capacity. The total volume of mixture was 3.00 ml, consisting of 0.30 ml of NADH solution, 2.40 ml of the substrate cocktail solution, 0.20 ml of the sample (or ammonia standard) solution and 0.10 ml of the enzyme solution. The NADH solution was prepared to be approximately 3×10^{-3}M NADH. The substrate cocktail contained the following concentrations: 3×10^{-3}M α-KG, 1×10^{-4}M ADP, and 1×10^{-4}M EDTA. The GDH solution was diluted, as obtained from Calbiochem, to obtain 100-150 I.U./ml. All solutions were prepared using the 0.05M, pH 7.8 potassium phosphate buffer as solvent.

The assay procedure consists of combining all solutions except the enzyme solution in the cuvette and recording the absorption due to NADH at 339 nm in a dual beam spectrophotometer. The enzyme was added, allowed to react 20 to 40 min, and the absorption due to NADH recorded again. The change in absorption at 339 nm was then related to the ammonia in the sample by comparison to the ΔA of an ammonia standard assay. One must also account for the twentyfold dilution of the fecal samples.

Delignification

Straw, ground to pass a 1 mm screen, (20g) is weighed and transferred to a 1 liter erlynmeyer flask. Distilled water (640 ml) is added to the flask which is then placed in a water bath which is adjusted to produce 70-80^{o}C inside the flask.

When the contents of the flask have attained reaction temperature, glacial acetic acid (2.0 ml) is added followed immediately by the addition of reagent grade sodium chlorite (6.0g).

A 150 ml erlynmeyer is then inverted into the neck of the reaction vessel which is maintained in the water bath at 70-80^{o}C for one hour. The addition of acetic acid and sodium chlorite are repeated hourly for a total of four hours.

After the final addition of acetic acid and sodium chlorite has been allowed to react for one hour, the flask is removed from the water bath and cooled to below 10^{o}C.

The holocellulose is filtered by vacuum with a minimum quanity of ice water to remove the color and ordor of chlorine dioxide. The filtered material is then air dried.

Because of the toxicity of the chlorine dioxide, the entire operation should be carried out in a well vented hood.

Determination of volatile acids (Acetate)

Five ml portions of digest or feed were placed in a centrifuge tube and then acidified with 1 ml of 25% metaphosphoric acid. This was mixed thoroughly, then allowed to stand for 30 minutes. The tubes were then centrifuged at 480 x g for 10 minutes. The supernatant was chromatographed directly or was first filtered through a 0.45μ millipore filter then chromatographed.

The supernatant were analyzed by gas chromatography (GC) using a Varian Model 3700 gas chromatograph equipped with a 6 ft x 1/8 inch glass column packed with 10% SP-1200/1% H_3PO_4 on Chromosorb W AW. The acids were detected with a flame ionization detector using helium as the carrier gas with a flow rate of 30 cc/min. The chromatograph was operated isothermally at 115°C. The retention times of the acids were compared to those of known acids.

Determination of CH_4 and CO_2 in Fermenter Gas

The fermenter gases were analyzed by gas chromatography (GC) using a Carle Model 8000 gas chromatograph equipped with a 6 ft x 1/8" stainless steel column packed with Porapak type N. The chromatograph was operated at 80°C with helium as the carrier gas. Gas pressure and flow rates were simply adjusted to provide adequate resolution of peaks. A thermal conductivity detector was used. Quantification was accomplished by measuring peak height, or alternatively using an electronic integrator.

A standard CH_4:CO_2 gas mixture was analyzed periodically to determine the correction factor needed to convert apparent (TC) ratio to the actual ratio. The data are reported as percent methane in the fermenter gas.

Dry Weight of Feed and Digest

Portions of either feed or digest were added to preweighed disposable plastic dishes and reweighed. These were dried in a 70°C oven overnight, then reweighed. The dry residue was expressed as a percentage of the wet weight of the feed or digest.

Total Oxidizable Carbon

The test specimen containing 0.25 g., or less, or cellulose and any amount of moisture and non-oxidizable contaminants is weighed into a 400-ml. beaker containing a 2-in. stirring bar, either glass-or plastic-enclosed. A volume of 25.00 ml. of 1.835N potassium dichromate solution is pipeted in, and the beaker is covered with a watch glass. While the mixture is rapidly stirred, 10 ml. of conc., reagent-grade sulfuric acid (d. 1.84) is slowly (15 sec) poured down the beaker spout from a graduate. After 15 sec. more, 30 ml. of additional acid is rapidly added, followed 3 min. later by 150 ml. of water. The excess dichromate may be titrated immediately or at any time later with ferrous ammonium sulfate.

The titration is preferably done electrometrically, without further dilution. Any commercial titrimeter, automatic if desired, suffices. The potentiometer is adjusted at the start so that microammeter reads zero. One drop of titrant at the end-point (whose approach is recognizable by color) gives a permanent deflection easily distinguishable from the usual slow drift or momentary fluctuations. This gives the titer, T.

The titrant, whose concentration does not appear in the equation, is compared with the dichromate in reference titrations at the beginning and end of a series of analyses. A reference titration is made by pipeting 5.00 ml. of the potassium dichromate solution into a solution which has just been titrated, and re-titrating to the same end-point. This gives T_r. Every different batch of sulfuric acid should be checked for blank by simulating the oxidation process, using 5.00 ml. of dichromate, 20 ml. of water, and the usual amount of acid and titrating as before. The difference between this and T_r is the blank, usually 0.03-0.0 ml., to be added to T.

Calculations

$$\% \text{ Cellulose} = 1.24V$$

where 1.24 is derived from 0.01240, the grams of cellulose equivalent to 1 ml. of 1.835N dichromate times 100%. V is the ml. of dichromate consumed per gram of test specimen, calculated as follows:

$$V = \{25.00 - 5.00\ (T/T_r)\}/W$$

where T is the titer of the dichromate remaining after oxidation, T_r is the reference titration, and W is the weight in grams of the test specimen.

APPENDIX B - BUDGET REPORT

Budget Proposed

Salary	$9,000.00
Benefits	600.00
Travel	500.00
Supplies	6,500.00
Indirect charges	2,880.00
TOTAL	$19,480.00

Budget Expenditures

Salary	$9,687.28
Benefits	1,013.07
Travel	98.50
Supplies	4,830.76
Indirect charges	3,173.29
Communications	4.10
Equipment	673.00
TOTAL	$19,480.00

Final Report for D.N.R.C.-A.E. Division

Content

I. Introduction - describing the overview of the research and the relative importance of each aspect.

A. lignin

B. CH_4 production

C. Summary of findings and the recommendations for implementing methane production.

II. Report of the Results

A. Cellulose and Wheat straw

B. Cellulose Fermentation Efficiency

C. Glucose and Glucose + Methanogenic Substrates

III. Conclusions

IV. Budget Report

V. Appendix - containing all figures and tables

I. Introduction

Today the world faces two important problems; an energy shortage and environmental pollution. This is of particular importance in the United States, a highly developed country. Many states are seeking ways to promote conservation of energy, alternative energy sources and methodology to avoid or abate pollution. Montana is somewhat unique in that, through the coal resources in the state we have inherited (or developed) a highly organized state agency to seek alternative energy resources. This is one of the best agencies in the USA for this mission. This project has been funded by the Renewable Alternative Energy Division of the state department of Natural Resources and Conservation. This project proposed to study the conversion of waste materials into fuel and/or "fuel sparing" products. Thus, the study has an aim toward both of the major problems mentioned above.

The objectives of this project are aimed at increasing the efficiency of conversion processes. While these objectives are not unique for Montana they are of great importance for any sparsely populated area. The economics for waste conversion are favorable in areas of dense population because of the large amounts of waste produced in a given region. Conversion processes can only be economically feasible for sparsely populated areas if the efficiency of the processes is increased.

The specific objectives that were set forth were to elucidate the limiting factors for conversion processes and to experiment with the chemical parameters to maximize conversion once the limiting factors were identified. The processes that we limited our attention to were those that would be adaptable to the needs and the types of wastes

produced in Montana.

The types of waste material produced in this state are predominantly from agricultural and municipal origin. Thus, we concentrated our attention to animal wastes and cereal straws. The processes we deemed most appropriate were processes involving biological conversion as opposed to industrial scale conversion. The latter are high technology processes, energy intensive and often produce pollutants which require additional processing for removal.

Plant material constitutes the largest fraction of waste material. Plant material is composed of pectin, hemicellulose, cellulose, protein, some lipid material and lignin. On a dry weight basis the hemicellulose and cellulose constitute approximately 70-75% of the mass. These substances can be converted to compounds which can serve as fuel or a chemical feed stock. In a chemical or biological process the cellulose and hemicellulose must be converted to simple sugars prior to conversion to a fuel or chemical feed stock. This process is impaired by the presence of lignin, which shields the cellulose and hemicellulose from chemical attack. This constitutes one of the limiting factors for efficient conversion of cellulose and hemicellulose to useful products.

A. Lignin

This proposal has included a study of a biological means of removing lignin from plant material. If a microbial strain can be found which can remove lignin, then plant material could be inoculated and incubated to remove the lignin and then the residue from this process could be used for production of fuel or other products.

At present there is no reasonable method available for monitoring the degradation of lignin. We have proposed a method of monitoring

lignin degradation. If this method works, then one can screen many microbial species for their ability to degrade lignin. The better species found could be used for testing the efficiency.

Not much progress has been made in this portion of the proposal due to a delay in funding and the state requirements of purchasing procedures. This has cost a six month delay in progress. Therefore the report of October covers most of our advances in this area.

B. Methane Production from Waste Plant Material and Animal Manure

Methane production from sewage or animal manures via anaerobic fermentation has been known to occur for over half a century. The basic processes involved have not been closely investigated until the past five years. This proposal has provided for an assessment of the impact of removing the lignin by measuring methane produced from plant material with and without its lignin content. Secondly, the efficiency of fermenting manure and cellulosic material mixtures was proposed. Fermentation of a mixture of this type was shown to yield a much greater quantity of methane.

In addition to the above studies an assessment of metabolic processes of the microorganisms involved in waste conversion to methane was also an objective. This was established as an objective because one objection to commercial production of methane by anaerobic fermentation was digester failure. In other words, it was currently thought the process was unreliable. This study investigated the causes of fermenter failure.

C. Summary of Objectives and Progress.

1. Lignin removal by microbial process: (no new progress since the last report).

2. Impact of lignin removal on efficiency of conversion. Approximately one and one half times as much energy can be converted if lignin is removed.

3. The most efficient mixture of cellulosic material and manure: The most efficient mixture was found to be 65% manure solids and 35% cellulosic material in the range of 6 to 10% total solids load.

4. Metabolic limitations:

   a. cellulose hydrolysis is rate limiting in high concentration loads.

   b. the energy load, when too high, can cause fermenter failure when the substrates are nonpolymeric or when the retention time of the digestion is too short.

II. Report of the Results

A. A comparison of fermentations of manure, and manure plus cellulose, or delignified wheat straw or wheat straw.

Wheat straw is a potential resource for conversion to useful products but lignin content imposes limitations. Lignin is a constituent of all vascular plants and is known to impair the natural breakdown of these materials (5, 6, 8, 9, 10, 13). Removing lignin improves the potential of utilizing waste plant material for production of paper (16), single-cell protein (2, 6), livestock feed (5, 6, 12) ethanol and methane. In this study a comparison of methane production from different manure loads was made with the methane production from manure plus wheat straw, manure plus delignified wheat straw and manure plus cellulose.

Varel et al. (15) studied the effects on methanogenesis for several concentrations of manure feed and several retention times (RT). The highest methane production (4.5 l/day-l) was obtained from feed stock of 8.2 percent volatile solids (VS) and a 3 day RT. Converse et al. (4) compared mesophilic and thermophilic fermentations at higher than normal feed loads. They found higher production rates from thermophiles but greater conversion from mesophiles. This investigation was concerned with methane production at higher than normal feed concentrations via added polysaccharides at 16 day RT in the mesophilic temperature range.

The three digesters used in this study were 3.8 liter glass bottles containing 3.2 liter volumes. Digesters were allowed to reach a steady state (3 RT's) on manure solids before any experiments were performed.

a thermal conductivity detector, and a 3.2 mm I.D. x 1.8 m stainless steel column packed with Porapak N (Waters).

The dairy manure was collected from a holding area within two hours of deposition. The manure composition is given in Table I; Appendix. Total solids (T.S.) and volatile solids (V.S.) were determined by standard methods (1). The fiber content was determined as described by Goering and van Soest (7) and the nitrogen content by the Kjehldahl method. The manure was mixed with tap water to bring it to the desired total solids content. This mixture was subjected to 30 sec. blending in a 3.9 l Waring blender at low speed, then packaged in 400 ml plastic bags and frozen for storage. This feed was thawed and stored at 4°C as needed.

A daily feeding regime was carried out in the following manner. The digesters were mixed using Flotec (F24P)-1062) pumps for 15 sec. prior to removing digesta. The digesta to be removed were pumped into a beaker, fresh feed was added and the digesters were again mixed for 15 sec. using the circulating pumps. (rate = 17 liters/min). Thorough mixing is necessary for good methane production. Feeds with added cellulose, delignified straw or straw were mixed just prior to feeding. Delignified straw was prepared by removing lignin with an acid chlorite treatment as described by Browning (3). Both straw and delignified straw were milled to pass a lmm screen.

The digesta removed were portioned for pH determination, dry weight, T.O.C.(11), ammonium ion concentration (14) and volatile acids. Prior to mixing the digesters and removing digesta the gas volume readings were noted and the methane and carbon dioxide content were determined.

This required approximately seven weeks. Higher loads were then introduced and fermentation maintained at that concentration for at least 16 days after the pH and gas production reached fairly stable values and rates. When this was attained the next higher load was introduced and the same procedure followed. Thus one digester received 5%, 6%, and 7% manure solids in subsequent order. The second fermenter received 5% manure, then 5% manure plus 1%, then 2% and finally 3% delignified straw. The third fermenter was treated like the second but cellulose (Whatman, standard grade W & R Balston) was used as the additional energy load. The first fermenter was also used for the fermentation of 5% manure plus 1% straw. All digesters were maintained at 37°C in a constant termperature bath. No attempts were made to control the pH during fermentation.

The pH values of digesta and gas volumes produced were determined daily. The pH was measured with a Corning 125 pH meter and the gas volumes were determined with GCA/Precision wet test meters, model 63111. Analyses were performed for ammonium ion concentration (14) and volatile acids 2 to 5 times per week. Volatile acids were determined by gas chromatography (G.C.) using a Varian model 3700 equipped with a Varian model 9176 recorder, a flame ionization detector and a 2mm I.D. x 1.8m. glass column packed with 10% SP-1200 acidified with 1% phosphoric acid. Total oxidizable carbohydrate (T.O.C.)(11) was determined on feed and digesta. The feed values were in the range of 80 to 84% ± 1% on a dry weight basis. The range for digesta was ca. 4% less.

The effluent gases were continuously passed through 125 ml gas sampling tubes (Supelco) and periodic analyses were done with a Carle model 8000 G.C. The G.C. was equipped with a Dohrmann SC 852 recorder,

The data from this study are summarized in Table II in the Appendix. The comparisons that can be made are the effects of increasing the energy load by adding carbohydrate as opposed to increasing manure solids, the comparable fermentation of delignigied straw compared to cellulose and most importantly, the effect of removing lignin, by comparing straw versus delignified straw.

The conclusion that can be drawn from this study are: 1. that ca 44% of the fermentable material in straw is shielded by lignin since the delignified straw produced 141% as much methane; 2. the methane production from added cellulosic material was markedly better than additional manure solids; and 3. the fermentations were stable as evidenced by the lack of accumulation of volatile acids.

B. Efficiency of Fermentation with Added Cellulose.

These experiments were designed to elucidate the maximum efficiency of fermenting cellulosic material mixed with manure. Since a biological system such as this can not produce commensuately higher methane yields with higher and higher energy loads, it is necessary to define the optimum mix for solid conversion to gas and methane yield. Our goal was to convert at least 30% of the solids (v.s.) and yet maximize the methane yield.

One question which had to be answered first was; is conversion of polymeric substrates to simple smolecules a rate limiting process? A comparison of fermentations of 6% cellulose plus manure and 6% glucose plus manure was made. The feeding regime was similar to the previous experiments described above. Smaller fermenters were used in this study, where the total ferment was 64 ml. Thus, for a 16 day R.T. only 4 ml were removed and added each day. The physical set up is shown in diagram 1 of the October 1978 report. The results of the fermentation are shown in figure 2 of the same report. The fermenter receiving 6% glucose failed within 7 days whereas the fermenter receiving 6% cellulose remained stable throughout 21 days.

At that time the experiment was terminated. It appeared from this experiment that cellulose hydrolysis was a rate limiting factor. This experiment has been repeated several times and fermentations up to 53 days have remained stable. Even with 7% cellulose the fermentation never indicated any instability.

The efficiency of fermentation of cellulosic material and manure was evaluated from experiments with 4, 5, 6 and 7% cellulose content. The experimental procedures remained the same as in previous experiments. The amount of methane per gram of volatile solids and per gram of added cellulose was calculated for each fermentation. These values were obtained from data gathered on 5 consecutive days after steady state (3 X 16 = 48 days) had been reached. These data are shown in Table III. From these data it was apparent that 4% cellulose added to the manure was the most efficient. Thus, 1, 2, and 3% levels of added cellulose needed to be assessed. But from this experiment and previous experiments with the lowerer levels it can be assumed that 3 or 4% added cellulose is the most efficient. This means that approximately 35% of the total solid content can contain added cellulose for maximizing methane production while maintaining a stable ferment.

There was a period during these fermentations where the gas production was more than twice the values given in Table III. We are curious as to why the gas production dropped off as it neared steady state. We feel this may be due to limitations in micronutrients, e.g. iron deficiency. This is worth investigation, since the peak methane production was found to be approximately 40% higher, i.e. 50 ml vs 80 ml.

C. Fermentation of Glucose and Glucose plus Methanogenic Substrates.

The following experiments were designed to test the limiting factors in the metabolism of the fermentative and/or methanogenic bacteria for methane production under high energy loads. It had been previouly shown that fermenter failure occurred under high energy loads as provided by manure and glucose mixtures but the addition of methanogenic substrates, viz. bicarbonate and acetate, had a sparing effect. (see the October 1978 report.) These experiments were essentially a repeat of those done previously with improved fermenter design to allow quantitative assessment of fermenter performance. The current hypothesis places the limiting process on methanogenic bacteria, however, from the observations of the accumulation of volatile acids we have proposed that under the special conditions of a high energy load in the form of soluble sugars the limiting metabolic processes lie with the fermentative bacteria. Namely, that the normal electron sink of molecular hydrogen production is incapable of regenerating enough oxidizing power (NAD). When this occurs the alternative electron sink of the "randomizing pathway" is utilized. This would account for the high accumulation of propionate over acetate in the previous experiments.

Fermentations of 5% manure solids with added glucose at levels of 1, 2, 3, 4, 5 and 6% were run. The fermenter performances are shown in figures 1 through 3. Fermenter failure is indicated by a decrease in pH, an increase in propionate and a decrease of methane production. Although the fermentation of 3% added glucose did not fail in the time allotted for the experiment, an accumulation of propionate was evident. This mixture

may have failed at longer time periods. The fermentations of 4, 5 and 6% added glucose all failed at 16, 12 and 8 days respectively.

To test the hypothesis stated above, two levels of glucose fermentation were chosen to which methanogenic substrates were added. The 2 and 6% levels were chosen. The maximum methane production in the latter 12 hr period of each day was determined for each fermenter. In the case of added glucose the production rate maximized at 30 to 32 ml for 2, 3, 4, 5 and 6% added glucose. (This is only shown in the figures for the 2 and 6% levels.)

If the metabolism of substrates by the methanogenic bacteria limited the overall production rate of methane, then this level could not be exceeded with added methanogenic substrates. Thus, three fermenters were set up to ferment 2% added glucose plus acetate, bicarbonate or hydrogen and a second set of three to contain 6% added glucose plus acetate, bicarbonate or hydrogen. Again, the experimental procedures followed were similar to all other experiments. Hydrogen was added by flushing the head space of the fermenter with H_2 gas twice each day at 12 hr intervals. The results of these experiments are shown in figures 4 through 6.

The most important finding here is that the maximum methane production for the latter 12 hr period of the day was 40 to 42 ml. This is about 150% higher than with glucose alone. This rate was found with glucose plus acetate. Thus the methanogens of the methanosarcina genus were capable of producing even more methane than when given glucose alone as an added energy source.

A surprising finding was the results with added bicarbonate. In previous experiments no accumulation of acetate was observed, while propionate accumulation was evident. In this experiment an appreciable acetate accumulation was noted with no appreciable accumulation of propionate. In fact it appeared as though the bicarbonate inhibited methane production form acetate. This conclusion can be drawn by comparing the methane production from 5% manure, figure 7, 5% manure plus 2% glucose, figure 1B, 5% manure plus 6% glucose, figure 3B, 5% manure plus 2% glucose and 4% bicarbonate, figure 4B and 5% glucose and 4% bicarbonate, figure 6A. The least methane was produced by 5% manure, 2% glucose and 4% bicarbonate. These results demand a rerun of these experiments.

It is also important to note that fermenter failure only occurred after 15 days in the fermenter receiving hydrogen and 6% glucose as an extra energy source. This indicates the stress put on the fermentative bacteria's ability to produce hydrogen. Methane was produced in all other fermenters even up to 24 days where as with 6% glucose in the previous experiments failure occurred within 8 days. These findings suggest that failure to produce methane was principally due to the metabolic limitations of the fermentative group.

III. Conclusions

The scientific conclusions for each of the experiments have heen drawn in the text of the results section. It is important to point out the impact that these conclusions have on the potential for producing methane fuel from anaerobic digestion of waste materials. This study has shown that lignin removal is an important objective for future research and development. In addition the results demonstrate the advisability of mixing cellulosic material with manures for greatly increased methane production and a failure free fermentation. The optimum mixture has been defined for manure and cellulose fermentation. These findings strongly suggest that a pilot plant study should commence to up hold these laboratory findings and to convince the engineering technologists of the failure free operation. The author is convinced that all haste should be made to implement these findings into production scale. The ideal situation would be to demonstrate the bioconversion of urban refuse (cellulosic material) and municipal sewage (manure) to methane for domestic fuel use. The author feels that now would be an appropriate time for the Renewable Alternative Energy Division and the author to jointly pursue federal funding to move the results of this three year study toward production of fuel on a larger scale.

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IV. Budget Report

1. Salaries and Benefits	20,498.00	18,143.29
2. Operating Expenses		
a. contracted services		0.87
b. supplies	3,000.00	3,890.45
c. communications		---
d. travel	---	---
e. rent		---
3. Equipment	2,660.00	
4. Administration	6,149.00	5,442.99
Total	32,307.00	27.477.60
Free Balance	4,829.40	

APPENDIX

Table I. Composition of Dry Matter of Dairy Cattle Waste

	% of dry matter
V.S.	83
Ether Extract	2.5-2.8
Cellulose	31
Hemicellulose	12
Lignin	12.2
Total Nitrogen (crude protein)	2.0 (12.5)
Ammonia	0.5
Volatile acids; acetic	0.1
propionic	trace
other	-

TABLE II. Fermenter Performance as a Function of Feed Composition[a]

	% Volatile Solids Destroyed (b)	pH	NH_4^+ Conc. mM Feed	NH_4^+ Conc. mM Digesta	$\%CH_4$	Methane production liters/day per liter digesta. (% control)[c]	
Manure 5%	30	7.8±0.1	55±3	53±2	57±0.5	0.40±0.03	(100)
Manure 6%	35	7.9±0.1	56±2	56±2	58±0.5	0.56±0.05	(140)
Manure 7%	34	8.3±0.1	58±5	57±4	58±0.5	0.66±0.16	(165)
5% Manure with 1% Cellulose	44	7.8±0.1	56±3	38±1	58±1	0.76±0.11	(190)
5% Manure with 2% Cellulose	48	7.9±0.1	55±3	44±2	56±1	0.94±0.08	(235)
5% Manure with 3% Cellulose	50	7.8±0.1	73±2	39±4	55±1	1.11±0.08	(278)
5% Manure with 1% Delignified Straw	45	7.8±0.1	56±3	41±5	58±1	0.76±0.08	(190)
5% Manure with 2% Delignified Straw	55	8.0±0.1	55±3	44±3	55±1	1.04±0.11	(260)
5% Manure with 3% Delignified Straw	56	8.0±0.1	73±2	51±1	54±1	1.22±0.14	(305)
5% Manure with 1% Untreated Straw	32	7.5±0.1	49±3	50±3	59±2	0.54±0.05	(135)

Footnotes a. Values reported are the mean value ± one standard deviation.

b. Calculated in the same manner as Varel et al. (15) viz. (moles CO_2 and CH_4 X 12) 100/0.4 (T.O.C.)

c. The percent figures in brackets allow comparison of the amount of methane produced as compared to the 5% manure solids fermentation.

Table III. Efficiency of Cellulose Fermentation.

Percent added Cellulose	Total gas per 24 hrs. (ml)	% CH_4	Vol. CH_4 (ml)	ml CH_4 per g cellulose	ml CH_4 per g V.S.	% Conversion of V.S.[2]
4%	86	55	47	188	147	37
5%	93	54	50	160	139	35
6%	97	52	50	133	125	33
7%[1]	122	50	61	157	139	37

1. 7% was not in steady state.

2. Assumed manure to be 80% V.S. and calculated as in Varel et al. ( ).

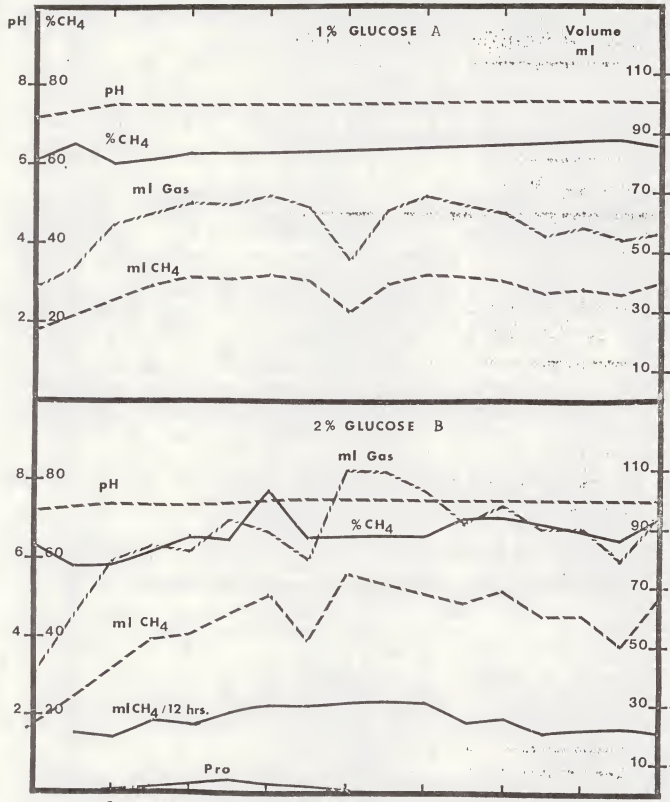


Figure 1 Methanogenesis from glucose-manure mixtures.

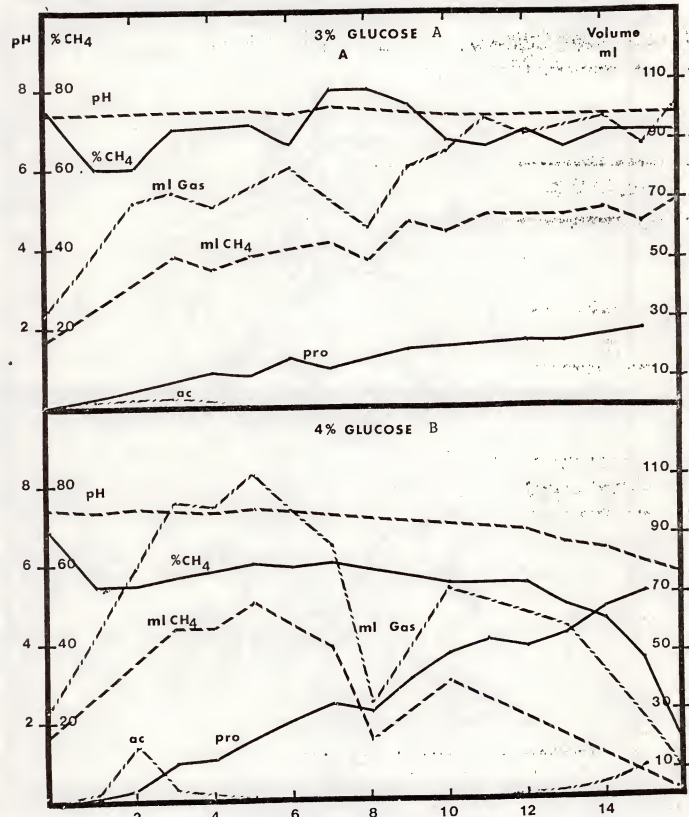


Figure 2 Methanogenesis from glucose – manure mixtures.

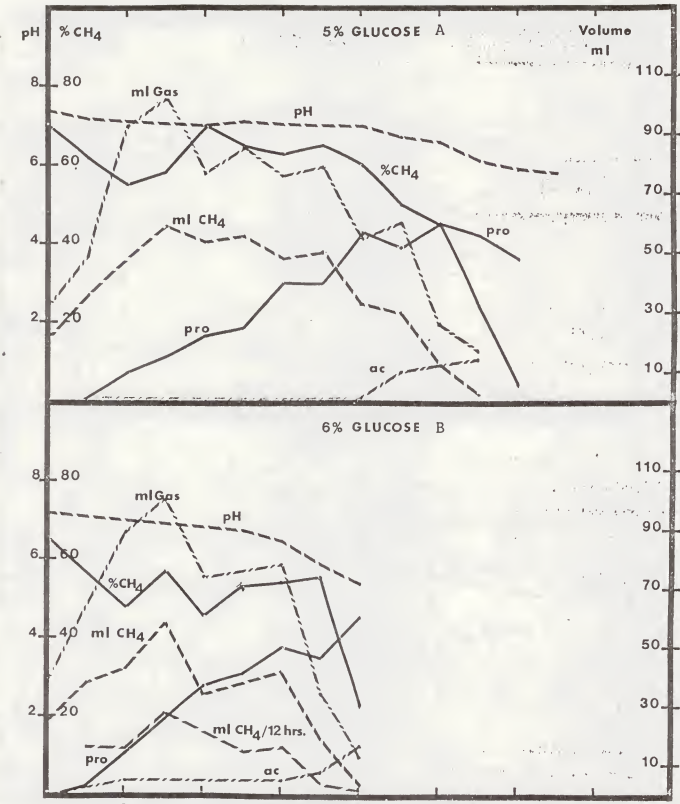


Figure 3 Methanogenesis from glucose - manure mixtures.

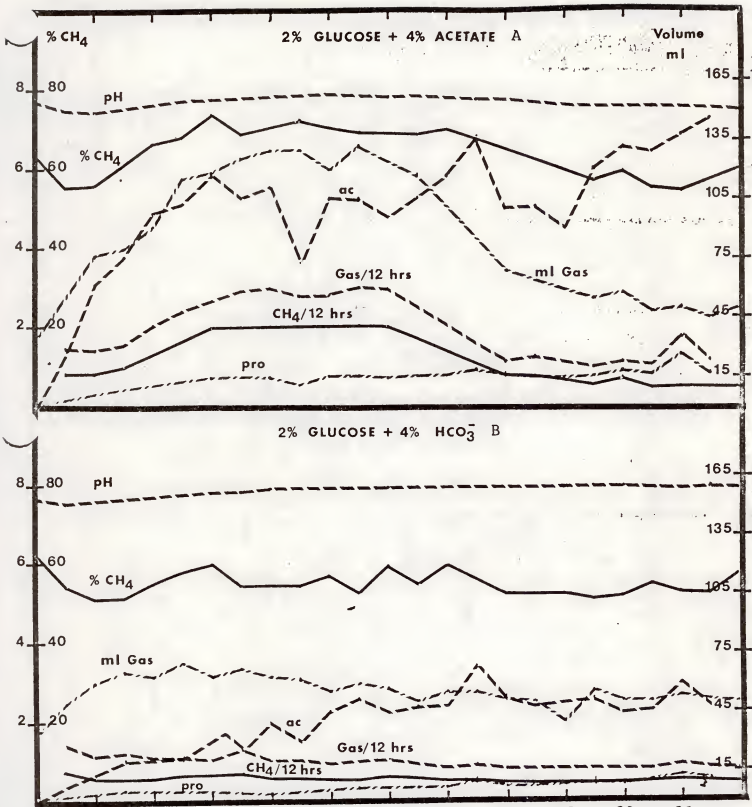
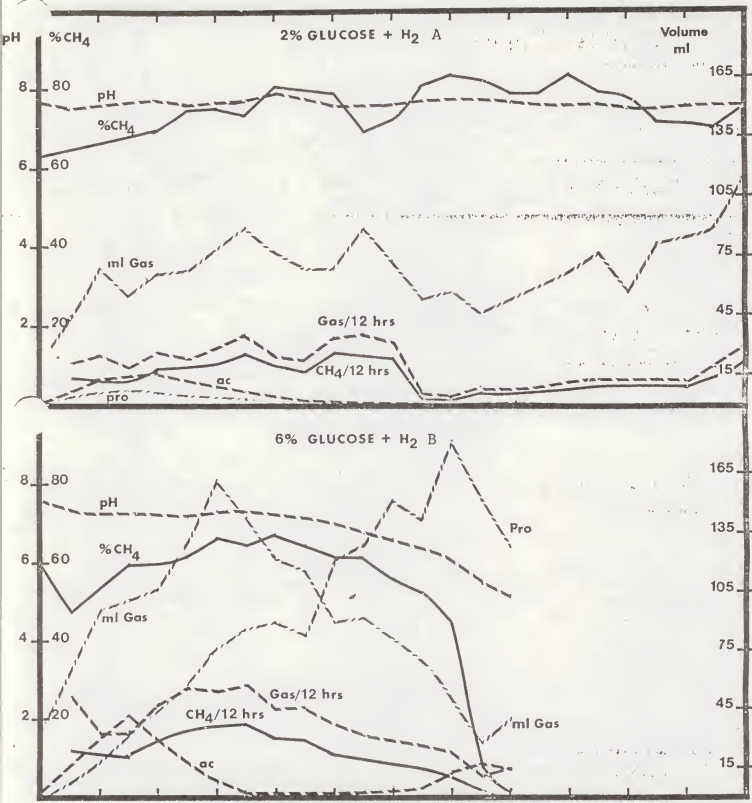


Figure 4 Methanogenesis from glucose and methanogenic substrates.



igure 5 Methanogenesis from glucose and methanogenic substrates.

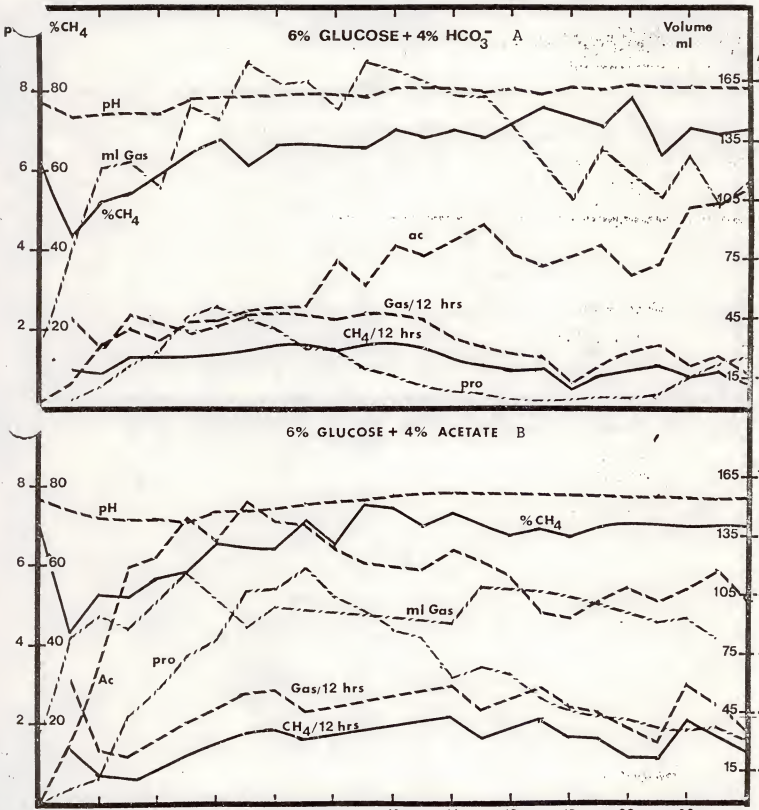


Figure 6 Methanogenesis from glucose and methanogenic substrates.

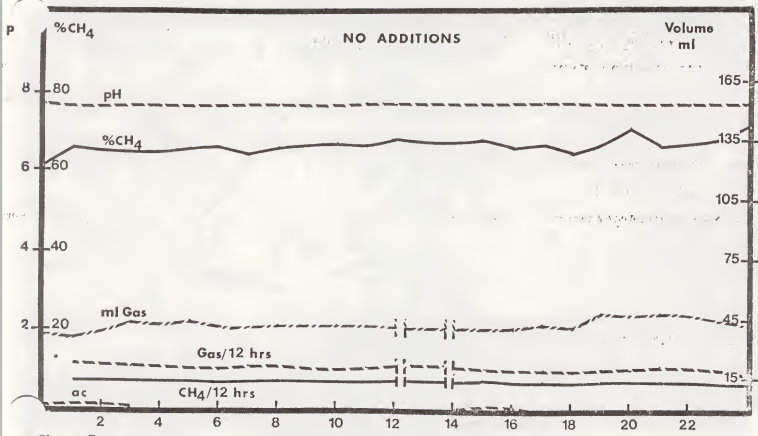


Figure 7 Methanogenesis from glucose and methanogenic substrates.

Final Report

for

DNRC Research Grant RAE 403-782

entitled

Bioconversion of Waste: An Attempt to Increase Efficiency

Time period April 1979-April 1980

by

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2. Carbohydrate and Manure Mixtures
a. cellulose + manure
b. glucose + manure; glucose & methanogenic substrates + manure.
3. Fertilizer Value

B. Summary of Results

II. Results

A. Lignin

B. Methane Production
1. cellulose + manure
2. effect of retention time
3. effect of micronutrients
4. effect of paper strip size

C. Stability
1. glucose
2. glucose + methanogenic substrates
3. amino acids and proteins

D. Fertilizer Value

E. Data Processing

III. Conclusions

IV. Budget Report

V. Appendix

A. Tables and Figures

B. Materials and Methods

I. Introduction

A. Summary of Objectives

1. Lignin removal. Studies on lignin have concentrated in three areas: A. Development of suitable high performance liquid chromatography (HPLC) assay for possible lignin metabolites: B. Examination of culture medium of Nocardia sp. for lignin degradation products: and C. Collection of several barley straws which may have altered lignin composition.

2. Mixtures of manure and cellulose and manure and sugars.

a. Cellulose and manure mixtures were digested anaerobically to produce methane. These fermentations were designed to further define the optimum mixture for methane production and solids destruction.

b. Sugar (viz. glucose) was fermented with manure to provide an energy load shock to digesters. Under these conditions the limiting factors and factors leading to digester failure would be most evident. It is important to identify the factors leading to digester failure in order to provide knowledge concerning stable digester operation. Compounds which serve as precursors for methane were added in several experiments in order to assess the impact the methanogenic activity would have on a digester that was heavily loaded with energy in the form of glucose. These experiments had implications on the advisability of designing two stage digesters in lieu of single stage digesters.

3. Fertilizer value. Assays were performed on the liquid and solid effluent of digesters receiving high nutrient loads and nominal nutrient loads. The object was to assess the impact on the nitrogen, phosphorus and potassium levels of residues from high energy loading rates.

B. Summary of Results

1. Lignin removal. A strain of Nocardia sp., a bacterium, isolated in Sweden was obtained for examination of lignin degradation. A lignin sample was prepared by periodate treatment of straw. The culture medium and an uninoculated control were assayed by the procedure described in the materials and methods section of the appendix. No significant differences were observed between the experimental and control media. At this time we are not sure of the means to resolve this approach.

2. Collection of barley straws with altered structural characteristics. We have an interest in the possibilities of finding a genetically altered plant which is low in lignin content or has an unusual lignin structure. These plants should be readily amenable to bioconversion techniques. A number of barley samples were taken from test fields by Mr. R. Eslick. These are to be tested for biodegradability and lignin content. We have not had the personnel or the time to process these samples.

3. Cellulose and manure mixtures in anaerobic digesters. Our tests in this category have expanded over our last report. We have varied the loading rate by changing the retention time (turn over time) as well as the loading concentration. Regardless of the retention time the optimum mixture for methane yield per amount of substrate remained in the region of 42 to 55% of the volatile solids as added cellulose. The solids conversion was optimized in this region also. These tests not only define the optimum mixture of manure and cellulose but they also suggest that high yields of methane will be produced from paper-manure mixtures. Along these lines we performed some experiments using waste paper. The waste paper was cut into several sizes in order to assess the effect

of the size of strips on methane production. The strip size varied from 0.1 cm to 1 cm widths. The results indicate there was no significant difference in methane yield over this range.

4. Anaerobic digester performance on high energy loads. Experimental digesters were fed manure and glucose and manure, glucose and methanogenic substrates. The objective of these experiments were to evaluate the advisability of pretreatment, such as hydrolysis; to give a high energy load so as to burden the rate limiting factors and to assess the impact of the methanogenic activity on the digester performance. These experiments indicate that pretreatments or two stage digesters are inadvisable, secondly, that the fermentative microbes of the digestion process may be the limiting step and thirdly, the methanogenic activity can be utilized to spare an unstable digester from failure.

5. The fertilizer value of anaerobic digester residues. Samples from digesters receiving high energy loads and digesters receiving normal energy loads were tested for nitrogen, phosphorus and potassium. The analytical results suggest neither the solid or liquid residue is a high grade fertilizer. The nitrogen content from high energy digesters to low energy digesters followed the trend as we predicted. The nitrogen content of the liquid effluent was 28-29% lower in the high energy digester and the nitrogen content of the solid residue was 22% higher in the high energy digester. This is presumably due to the greater cell mass in the high energy digester.

II. Report of Results

A. Lignin

Studies on lignin have been concentrated in three areas: (1) Development of a suitable high performance liquid chromatography (HPLC) assay for possible lignin metabolites, (2) Examination of culture medium of Nocardia sp. for lignin metabolites and (3) Collection of several barley straws with possible altered lignin.

1. Development of HPLC assay for lignin metabolites. Four compounds which are thought to be metabolites of lignin were used as test standards. Assays covering a range of conditions were found to be suitable for separating these compounds. This is useful, since experiments examining lignin degradation products may contain other lignin metabolites which may not separate under some of these studied conditions but may separate in others. It is possible that none of these conditions may be suitable if a large number of similar metabolites are formed from lignin. Therefore these assay conditions are regarded as a guide which may be modified if experimental conditions dictate.

2. Examination of Nocardia sp. culture medium for lignin metabolites. A strain of Nocardia thought to possess lignin degrading ability was cultured on lignin isolated by periodate treatment of straw. The culture medium and an uninoculated control were assayed by the procedure described in section A. No significant difference was observed between the incubated and uninoculated culture media.

3. Collection of barley straws with possibly altered lignin. Professor Eslick of the Plant and Soil Science Department at Montana State University has observed several strains of barley which have unusual straw characteristics. He believes some of these varieties may contain less lignin or altered lignin structure. Such plants are of obvious interest to our project. These straws may be more amenable to biodegradation of lignin or perhaps may be sufficiently low in lignin that the polysaccharides are more readily hydrolyzed directly. We have collected samples of some of these varieties of various times throughout the growing season. When time and resources permit, these samples will be examined for ease of polysaccharide hydrolysis and possible lignin content.

B. Cellulose Additions

1. 16 day retention time, 37°C. Seven 64.0 ml fermenters were used in this experiment. Each day the fermenters received 4.0 ml manure containing 5% total solids. They also received 0, 40, 80, 120, 160, 200 and 240 mg powdered cellulose each day. This corresponded to 0, 1, 2, 3, 4, 5 and 6% added cellulose. Methane production for the 0 (control), 2, 4 and 6% cellulose additions are shown in Figure 1 . Graphs for 1, 3 and 5% added cellulose are not shown, but values are between 0 and 2%, 2 and 4%, and 4 and 6% respectively, and follow the general pattern shown for 2, 4 and 6% added cellulose. Volatile acids did not accumulate in these experiments, and no major shift in pH was observed. The initial pH of the fermenters ranged from 7.6 to 7.8, and the final pH ranged from 7.5 in the control and 1% fermenters, through 7.4 in the 2, 3, 4 and 5% fermenters to 7.3 in

the 6% cellulose fermenter. Figure 1 shows that daily methane production is irregularly cyclic, and the amplitude of the oscillations become more pronounced as the % added cellulose increases.

2. 16 day retention time, 37°C, micronutrient additions. The above experiment was repeated for 0, 2, 4 and 6% added cellulose, except each fermenter received the following micronutrients: $FeSO_4 \cdot 7H_2O$ (11 mg/l), $CuSO_4$ (0.05 mg/l), $MgCl_2 \cdot 6H_2O$ (173 mg/l), all other parameters were similar to the above experiment. The results were similar to those of the above experiment. Methane production was similar and was cyclic, especially at the higher feeding rates. Volatile acids did not accumulate, and pH did not show any significant change. The addition of these micronutrients did not change methane production, the cyclic nature of methane production, volatile acid profiles or pH

3. 8 day retention time, 37°C. Four 64.0 ml fermenters received daily feedings of 8.0 ml manure containing 5% total solids. They also received daily 0, 160, 320 or 480 mg powdered cellulose. This was 0, 2, 4 or 6% added cellulose. Total gas and methane production are shown in Figures 2, 3, 4 and 5. The total gas and methane production from the fermenters receiving 4 and 6% added cellulose show a definite cyclic pattern similar to those described in Experiments 1 and 2. Volatile acid profiles for the fermenters are shown in Figure 6, 7, 8 and 9. Volatile acids were observed in the control fermenter, however the concentration of these acids declined throughout the 30 days of the experiment. All of the fermenters were maintained on an 8 day RT for 3 full retention times prior to beginning the experiment. The observed steady decrease in acid concentration in the control fermenter suggests the accepted time to reach steady

state conditions may not always be valid. The concentration of volatile acids in the 2% and 4% added cellulose fermenters increased initially, then decreased to very low levels. This suggests that initially volatile acid production by the fermentative bacteria exceeded the ability of the methanogenic and acetogenic bacteria to utilize these acids. The concentrationof volatile acids in the fermenter receiving 6% added cellulose was significantly higher than the concentrations in the other fermenters. The pH profiles of the fermenters are shown in Figures 10, 11, 12 and 13. The pH of the control fermenter remained nearly constant at 7.28 throughout the experimental period. The pH of the experimental fermenters decreased to 7.16, 7.10 and 6.91 for the 2, 4 and 6% cellulose additions respectively. There appears to be some correllation between pH and concetration of acetate in the fermenter receiving 6% added cellulose.

4. 4 day retention time, 37°C. Four 64.0 ml fermenters received daily feedings of 16.0 ml manure containing 5% total solids. In addition they received each day 0, 320, 640 and 960 mg powdered cellulose, corresponding to 0, 2, 4 or 6% added cellulose. Methane production from these fermenters is shown in Figures 14, 15, 16 and 17. Methane production increased with time in the control fermenter as well as the experimental fermenters. Methane production in the control fermenter was around 25 ml/day initially, around 45 ml/day at the conclusion of the experiment, and appeared to be more or less constant for the final eight days of the experiment. Again this suggests that stabilizing the fermenters for three retention times prior to beginning the experiment does not allow sufficient time for the fermenters

to attain steady state. The profile for methane production for the fermenter receiving 2% added cellulose was similar to that of the control fermenter. The average daily methane production increased from around 20 ml/day to around 60 ml/day. Methane production in the 4% and 6% added cellulose fermenters increased during the duration of the experiment, but remained variable, that is the methane production did not stabilize at some value. The pH profiles of the fermenters are shown in Figures 18, 19, 20 and 21. The pH of the control fermenter increased from 7.04 to 7.13 during the experiment, again suggesting that the fermenters were not in steady state at the beginning of the experiment. The pH profiles of the 2% and 4% added cellulose fermenters are characterized by a dramatic decrease during the first eight to ten days of the experiment. The pH then increases over a seven day period to a steady state value of approximately 6.97 and 6.87 for the 2% and 4% added cellulose fermenters respectively. The pH profile for the 6% added cellulose fermenter is more complex. The pH decreases for the first seven to eight days, then cycles irregularly throughout the remainder of the experiment. The volatile acid profiles of the fermenters are shown in Figures 22, 23, 24 and 25. The concentrations of acetate and butyrate were highest early in the experimental period and decreased with time. Small and generally constant values were observed for Valerate. Propionate increased initially in the control and 2% added cellulose fermenter, but levelled off or increased slightly by the end of the experimental period. Propionate definitely increased initially in the 4 and 6% added cellulose fermenters, and the propionate concentration was variable in the 6% fermenter.

5. Effects of paper strip width on fermenter performance; 16 day RT, 37°C. Waste paper is an excellent source of energy for anaerobic digestion. This experiment was designed to test the effects of paper widths which could be easily generated by shredding, on fermenter performance. One percent paper, or powdered cellulose was added along with the daily feeding of 5% solids manure. The gas production was determined by averaging the daily values for days 17 through 48.

Gas Production from Added Cellulosics

Fermenter	Average Daily Gas Production
1% added paper, 1.0 cm strips	52 ± 5 ml
1% added paper, 0.5 cm strips	54 ± 4 ml
1% added paper, 0.25 cm strips	57 ± 7 ml
1% added paper, 0.1 cm strips	54 ± 12 ml
1% added powdered cellutose	54 ± 4 ml
Control, no additions	39 ± 3 ml

All of the cellulosic additions significantly increased the production of gas above that of the control fermenter. There was no significant difference between the gas production from the various paper strips or cellulose.

C. Stability

We recently reported increased methane yields from cattle manure-cellulose mixtures and manure-delignified straw mixtures (21). In this study we examined the effects on methane yields and volatile acid production by different energy loads in the form of soluble sugar, or sugar plus methanogenic substrates. This approach permitted an inspection of the changes in metabolic activity of this ecosystem and an evaluation of the rate limiting factors in methanation under these conditions.

I. The effects of added glucose on anaerobic digestion: A. Methane production.

Seven fermenters were established as described above to assess the amount of glucose which caused failure. The fermenters received 5% by weight manure solids and 0, 1, 2, 3, 4, 5 and 6% additional glucose by weight. Methane production for one of three experimental trials is depicted in Fig. 26. In all trials the methane production was similar for each glucose level and failures for a given glucose level occurred within a 24 hour period.

Methanation ceased (fermenter failure) and pH decreased in fermenters that received 4, 5 and 6% added glucose. Methane yield fell below 4 ml/day on days 16, 11 and 8, respectively. The fermenters that received 1, 2 and 3% glucose remained productive and pH was stable.

B. Volatile acid production

The profile of acetate concentration for the fermenters receiving 0, 1, 2, 3, 4, 5 and 6% glucose is shown in Fig. 27. Acetate did not accumulate until one or two days prior to cessation of methanation.

Digesta pH values at the time acetate began to accumulate were near 7. The propionate profiles are depicted in Fig. 28. The rates of accumulation are proportional to the glucose concentration. Propionate accumulated in all fermenters but decreased to nondetectable levels in fermenters that received 1 and 2% added glucose. Propionate reached the highest concentration in fermenters receiving 4% glucose and propionate concentration began to decrease in fermenters receiving 5% glucose just prior to failure at a rate exceeding the wash-out.

C. Hydrogen production

Head space hydrogen was monitored in fermenters receiving 5% manure alone and manure plus 6% glucose. We felt that if H_2 accumulated it would be most evident in fermenters that received the highest energy load. The H_2 levels were, at best, only detectable. The H_2 level in 6% glucose experiments did not increase and if anything decreased, i.e. became virtually undetectable. The H_2 level in fermenters receiving manure only was small but significantly greater than that in the 6% glucose experiments.

II. The effects of glucose and methanogenic substrate additions on anaerobic digestion: A. Methane Production.

Two and six percent added glucose were chosen as representative of stable and unstable fermentations as demonstrated in the previous experiments. Methane production from glucose and methanogenic substrates is shown in Fig. 29. The greatest methane yield was obtained from 6% glucose and 1% acetate (20.8 mM and 10.6 mM fermenter concentration, respectively). The acetate addition improved the methane yield at both 6 and 2% glucose concentrations. The methane production from 6% glucose and 1% acetate leveled off at 150 ml per day after 20 days

(Fig. 29 includes only the first 16 days). The addition of methanogenic substrates provided methane production throughout the experimental period in contrast to fermentation of glucose alone. This is readily seen from plots 5, 6, 7 and 8 (Fig. 29) which received 6% glucose; 6% glucose and 1% acetate; 6% glucose, H_2 and 2% HCO_3^-; and 6% glucose, 1% acetate, H_2 and 2% HCO_3^- respectively. Secondly, the consistent yield from the control, (5% manure solids) plot C, indicated the fermenter design and the daily manipulations provided a mechanically sound and reproducible procedure. Thirdly, it is interesting to note that methane production from glucose and acetate (plots 2 and 6) was higher than from glucose, acetate, H_2 and HCO_3^- (plots 4 and 8).

Additional experiments (data not shown) were performed with glucose and singular additions of methanogenic substrates. Experiments with 4% HCO_3^- and either 2% or 6% glucose yielded slightly less methane than experiments depicted in Fig. 29 that included HCO_3^- and H_2. These fermentations also remained productive throughout the experimental period. Experiments with H_2 as the sole methanogenic substrate showed methane yields were unaffected in 2% glucose fermentation, whereas, at 6% glucose the fermentation failed after 15 days. This fermentation lasted 5 days longer than 6% glucose alone. The experiments with different levels of acetate all produced methane throughout the 24 day period. The 6% glucose, 4% acetate was highly variable and the average daily yield was 58 ml CH_4 per day compared to 103 ml CH_4 per day for 6% glucose, 1% acetate over the same period. The methane yields for the 2% glucose plus 1% or 4% acetate were both ca. 65 ml per day.

B. Volatile acid profiles

Fig. 30 depicts volatile acid profiles for fermenters receiving 6% glucose and methanogenic substrates whose methane volumes are shown in Fig. 29. The dramatic accumulation of propionate that occurred in fermenters that received 6% glucose did not occur in fermenters that received 6% glucose plus methanogenic substrates. When fermenters received 2 or 4% acetate and 6% glucose, propionate accumulation was similar to fermenters receiving 6% glucose alone (data not shown). Acetate accumulated in fermenters that received HCO_3^- (Fig. 30 C and D) and in experiments where HCO_3^- was the sole methanogenic substrate added with glucose.

The volatile acid analyses on fermenters receiving 2% glucose and methanogenic substrates showed accumulation of acetate in fermenters receiving H_2, HCO_3^- and acetate collectively. The levels of acetate reached a maximum of 0.17%. The volatile acid profiles in all the other fermentations at the 2% glucose level were comparable to fermentations of 5% manure alone.

3. The effects of amino acid or protein additions on methane production. The purpose of this experiment was to assess the effects of proteinaceous material on fermenter performance. Most of Montana's agricultural wastes are not high protein wastes, but a few specific types such as cheese factory wastes and slaughter house wastes would contain significantly higher protein. Therefore protein or amino acids were added to anaerobic fermenters in 1, 2 and 3% increments. All other fermenter parameters were similar to those described in the cellulose experiments.

III. Results and Discussion

Methane production from fermenters receiving amino acids or protein initially increased upon addition of substrates, but showed little increase over control by the end of the 53 day experimental period (Figure AAP 1 and AAP 2). The data for methane production is summarized in table AAP 1. This data must be interpreted with caution because the methane production decreased over the experimental period for the fermenters receiving amino acid or protein. This suggests that a steady state level of methane production was not attained. An examination of the pH profiles for the fermenters indicates that the pH increased over the control for all additions.

The addition of carbohydrates to fermenters normally results in a decrease in pH. The increase in pH in these experiments is probably due to an increase in ammonium ion concentration resulting from catabolism of the amino acids or proteins. The modest change in pH seen here would probably not, by itself, seriously affect methane production.

D. Fertilizer Value

The digest was collected from two fermenters, one receiving 5% total solids manure and the other 5% total solids manure plus 3% added glucose. Each digest sample was centrifuged to yield a moist solid pellet and a turbid supernatant. Each of these four samples were analyzed for % nitrogen, % total P_2O_5, % available P_2O_5, % total K_2O and % soluble K_2O.

Sample	% Nitrogen	% total P_2O_5	% available P_2O_5	% total K_2O	% soluble K_2O
Digest pellet	2.27	2.06	1.69	1.49	1.56
Digest pellet (3% glucose)	2.75	1.89	1.35	1.17	1.15
Digest supernatant	0.07	0.03	< 0.01	0.16	< 0.01
Digest supernatant (3% glucose)	0.05	0.03	< 0.01	0.16	< 0.01

The addition of 3% glucose to the manure resulted in no significant change in the fertilizer properties of the supernatants. The digest pellet from the fermenter receiving the 3% glucose was somewhat higher in nitrogen but lower in categories of P_2O_5 and K_2O.

E. Computerization of Data

Portions of our research time during this year have been dedicated to the rapid and accurate processing of data obtained from our fermentation studies. Several thousand individual data points have been collected during these studies. Simple hand processing of these data is time consuming. In the past raw data was first recorded, then calculations performed to yield data suitable for reporting. Individual parameters were then graphed individually, or in some cases collectively.

Each graphing operation required significant portion of time which limited the number of graphs which could reasonably be produced. The metabolic interactions which occur in these anaerobic fermentations are complex. Simple examinations of the raw data or tabular processed data do not always reveal these interactions. Data graphs often illustrate relationships not readily seen in tables.

Processing and graphing data can be performed more accurately and quickly by use of computers. Many of the figures included in this report were produced with the aid of a computer. Much less time was required than would have been used in hand production. Cost was reduced since a draftsman was not required. The probability of accuracy was increased because the number of times the data was manipulated by people was reduced. We believe the time we have spent establishing a procedure for computer processing our data will significantly improve the overall efficiency of our studies.

III. Conclusions

A. The Optimum Mixture of Cellulose and Manure

The optimum mixture of cellulose and manure was found to be in the range of 42 to 55% of the convertible solids as added cellulose. The total solid concentration was between 8 and 10% for this range. In addition to mixture composition different retention times (RT) were also investigated. The retention times used were 4, 8 and 16 days. Interestingly the optimum mixture range did not change. The efficiency of conversion dropped with shorter retention times. The amount of solid conversion was considerably higher with the 16 day

RT. Thus, it is our recommendation that RT's near 16 days be used. Finally as an extra effort we ran some experiments on manure-waste paper mixtures varying the size of the paper strips. We demonstrated the strips could be as large as 1 cm (0.4 in) with no significant loss in conversion efficiency. Further, the waste paper yielded methane at the same rate as pure, powdered cellulose. This indicates waste paper is an excellent source of cellulose for anaerobic conversion to methane.

B. Anaerobic Digester Performance on High Energy Loads

These experiments demonstrated the predominance of accumulation of propionate over all other volatile acids. Acetate did not accumulate in digesters stressed with high energy loads until just prior to failure. These findings clearly suggest that acetate utilization by methanogens is not a rate limiting factor. Furthermore, the addition of acetate along with high energy loads in the form of glucose showed a beneficial effect on digester performance. The other methanogenic substrates H_2 and CO_2 also showed a sparing effect but not as dramatic as acetate. It is imperative to establish whether or not the methanogenic activities of acetate splitting or H_2 oxidation are able to keep pace with the fermentative bacterial activities. These experiments suggest that fermentative activity in conjunction with the acetogenic activities are more critical than the methanogenic metabolism. If this can be clearly demonstrated then the recommendations of two stage digestion or hydrolytic pretreatments are clearly in error.

The sparing effect of the acetate additions to stressed fermenters could also suggest that enrichment of the methanogenic microbial

population could have occurred. This would allow a greater flow of acetate and H_2 to methane formation. This seems to be less likely to be responsible for all the observations since under high energy loads greater amounts of acetate should have been produced unless there was a change in the metabolism of the fermentative class of bacteria. This we think is most probable. Further study is needed to resolve these dilema.

C. Fertilizer Value

The fertilizer value of the effluent was evaluated and found to be a good, but low grade, fertilizer in the major nutrients, nitrogen, phosphorus and potassium. We believe the value of the effluent as a soil conditioner is very high. Thus, we recommend the use of the solid residue as a plant fertilizer and soil conditioner.

IV. Budget Report — 1980-1981 Grant Period

Proposed Budget	
Salaries and Benefits	$18,924
Equipment	4,000
Administration	5,677
Total	$28,601
Expenditures	
Salaries and Benefits	$18,359.76
Equipment	4,516.16
Administration	5,507.91
Total	$28,383.83
Balance	$217.17

B. Materials and Methods

1. Manure and Digest. Manure was collected from a concrete holding area at the Montana State University dairy within 2 hrs of deposition. The manure was mixed thoroughly, then packaged in plastic bags and frozen. This procedure provided a homogeneous substrate for several related experiments. The bags of manure were thawed as needed, diluted with tap water to 5% total solids (4.8 to 5.2%) blended for 30 sec in a 3.8 l waring blender, then repackaged and frozen in 1l bags. The 5% solids manure was thawed and stored at 4° as needed. The manure composition is shown in Table 1. Total solids (T.S.) and volatile solids (V.S.) were determined by standard methods (1). The fiber content was determined as described by Goering and van Soest (7) and the nitrogen content by the Kjehldahl method.

The digest used in starting the experimental fermenters was obtained from a larger digester which had been maintained at 37° on a 16 day retention time (RT) for over two years on manure containing 5% total solids.

2. Fermenters. The fermenters were made from 100 ml wide mouth lyophilization flasks (Thermovac) fitted with a #9 three hole rubber stopper (Fig. 1). One hole was fitted with a y-tube for gas sampling and gas collection. The gas was collected in a calibrated gas receiving vessel. The second hole was fitted with a 10 ml syringe to allow additions of feed and removal of digest. The third hole was normally plugged and opened only during feeding and removal of digest to allow pressure equilibration. The working volume was 64 ml.

3. Conditioning and daily procedure. The fermenters used in 16 day RT experiments were maintained with 5% total solids manure as feed until pH, gas volume and gas composition were similar in all fermenters. The fermenters used in the 8 day RT and 4 day RT experiments were inocculated with digest from a 16 day RT fermenter, then fed 5% total solids manure on an 8 day RT or 4 day RT regime for three retention times. Following this conditioning period, the experiment was initiated when pH, gas volume and gas composition were similar in the fermenters. All experiments were performed in a water bath at 37°. The fermenters were then fed 5% total solids manure plus 0, 1, 2, 3, 4, 5 and 6% (% of daily feed) additional solids as cellulose powder or computer paper of varying widths. In the remainder of the text we refer to added substrates in terms of percent added dry weight. Each day the gas volume and composition were determined as described below. The fermenters were swirled to suspend particulates, 4.0 ml, 8.0 ml or 16.0 ml digest was removed and 4.0 ml, 8.0 ml or 16.0 ml of the appropriate feed was added. After resealing, the fermenters were again swirled to mix the feed and digesta. The pH and volatile acid composition were determined on each digest sample. No attempts were made to adjust pH.

4. Analyses. Gas volume was determined by water displacement using acidified water. Gas composition and volatile acid profiles were determined by the gas chromatographic procedures described by Robbins et al. (21). Gas volume and composition were determined every 12 hrs or daily as needed.

5. Chemicals. Cellulose Powder (Whatman CF11) was alkalai treated at room temperature for 1 hr in 0.1 N NaOH. It was then washed with water until the wash water was neutral. The residue was dried at 75° overnight. Computer paper was cut into strips of 10 mm, 5 mm, 2.5 mm and 1 mm widths. The amounts added are described for each experiment.

6. Conditions for HPLC assay.

Temperature: Ambient

Column: 150 x 4.6 mm column containing 2 μm particles of LC-18 obtained from Supelco, Inc.

Solvent: Initially pure water changing with time to 90% acetonitrile, 10% water.

Solvent, rate of delivery: Suitable separation at rates varying from 1.2 ml/minute to 1.5 ml/minute.

Time: 20 minutes gives good separations in a reasonable time period. Other times may be acceptable.

Solvent program shape: Linear increase in acetonitrile from 0% to 90% was acceptable. Also acceptable was a concave program which increased the percentage acetonitrite slowly early in the program. This program also ranged from 0 to 90% acetonitrile.

Recycle time: It was noted that a minimum of 10 minutes was required to recycle column to starting conditions. This could be accomplished by reversing the program in 10 minutes. An additional wait of 5 to 10 minutes was sometimes desirable.

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Table 1. Composition of dry matter of dairy cattle waste.

	% of dry matter
Volatile solids	83
Ether extract	2.6
Cellulose	31
Hemicellulose	12
Lignin	12.2
Total nitrogen (crude protein)	2.0 (12.5)[a]
Ammonia	0.5
Volatile acids	
Acetic	0.1
Propionic	Tr
Other	-[b]

[a] Data in parentheses indicate total nitrogen x 6.25 = crude protein.

[b] Not detected.

Table 2. Comparison of Average Daily CH_4 Production and Percent Conversion.

% Cellulose(g)	g VS	ml CH_4	ml CH_4/ g cellulose	ml CH_4/ g VS	% conversion[a]
		—— 16 d RT[b] ——			
0 -	0.17	25 ± 2	-	150 ± 12	30
1 (0.04)	0.21	36 ± 4	280 ± 50	170 ± 19	36
2 (0.08)	0.25	47 ± 5	280 ± 38	190 ± 20	42
3 (0.12)	0.29	61 ± 7	300 ± 42	210 ± 24	49
4 (0.16)	0.33	70 ± 10	280 ± 50	210 ± 30	50
5 (0.20)	0.37	80 ± 16	280 ± 70	220 ± 43	54
6 (0.24)	0.41	84 ± 14	250 ± 50	200 ± 34	51
		—— 8 d RT[c] ——			
0 -	0.33	44 ± 2	-	130 ± 6	25
2 (0.16)	0.49	69 ± 3	160 ± 6	140 ± 6	33
4 (0.32)	0.65	98 ± 9	170 ± 22	150 ± 14	38
6 (0.48)	0.81	92 ± 13	100 ± 23	110 ± 16	29
		—— 4 d RT[d] ——			
0 -	0.66	47 ± 3	-	71 ± 5	17
2 (0.32)	0.98	59 ± 16	38 ± 16	60 ± 8	15
4 (0.64)	1.30	102 ± 18	86 ± 23	78 ± 14	21
6 (0.96)	1.62	91 ± 17	46 ± 15	56 ± 10	16

[a] Percent conversion = $12(\text{mol gas}) \cdot 0.4^{-1}(\text{g VS})^{-1} \cdot 100$

[b] Daily averages were computed using data from days 17 through 56.

[c] Daily averages were computed using data from days 17 through 30.

[d] Daily averages were computed using data from days 16 through 36.

Table AAP 1. Methane Production from Protein and Amino Acid Additions.

Average Volume Methane	Control	1% AA addition	2% AA addition	3% AA addition	1% Pro addition	2% Pro addition	3% Pro addition
ml/day	26	30	35	40	34	42	39
gram(per day) added substrate	0.00	0.04	0.08	0.12	0.04	0.08	0.12
ml CH_4 $g^{-1}day^{-1}$	-	100	113	117	200	200	108

AA - amino acid; Pro - Protein

ml CH_4 $g^{-1}day^{-1}$ was determined by subtracting the volume CH_4 produced by the control fermenter from the total volumn produced by each experimental fermenter. This difference was the volume CH_4 produced from the added substrate.

Average Methane Production/Day (day 11-53)

	1% AA	2% AA	3% AA	Cont	1% P	2% P	3% P
11	29	41	44	26	38	41	39
12	26	40	44	26	39	37	37
13	30	38	44	26	40	38	36
14	29	40	45	28	39	38	38
15	32	39	42	28	38	37	38
16	32	40	41	26	37	37	42
17	37	45	42	24	37	40	42
18	37	41	37	26	38	39	44
19	35	37	36	23	38	41	44
20	38	40	37	25	38	44	47
21	36	40	38	26	39	44	48
22	33	42	38	26	34	45	46
23	30	37	40	24	39	44	48
24	29	36	37	19	34	45	43
25	32	35	40	24	35	46	43
26	30	36	40	25	34	42	41
27	29	35	39	25	33	41	41
28	35	37	42	26	33	50	41
29	30	33	38	26	31	41	41
30	34	35	41	30	33	41	41
31	30	34	41	26	31	39	41
32	30	36	42	26	33	41	40
33	33	35	-	26	31	46	43
34	18	33	39	24	33	51	40
35	26	35	39	24	32	47	39
36	26	35	38	25	33	44	37
37	30	35	44	26	37	48	38
38	29	39	41	25	35	42	36
39	26	36	39	26	35	41	37
40	26	34	38	26	32	43	-
41	20	29	36	20	31	33	-
42	19	27	35	29	31	35	31
43	23	27	37	26	31	42	33
44	33	28	41	26	34	39	35
45	26	25	43	37	33	44	34
46	30	31	42	29	36	44	37
47	25	30	39	26	33	37	36
48	28	30	41	30	33	43	37
49	27	31	40	26	26	40	36
50	27	30	34	28	23	42	33
51	31	27	40	33	21	42	32
52	40	35	45	32	28	45	35
53	30	34	38	29	24	44	33
Total	1276	1503	1677	1134	1443	1803	1602
Average	30	35	40	26	34	42	39
Days	43	43	42	43	43	43	41
ml CH_4 above Control	4	9	14	0	8	16	13
g AA or Protein Added	0.040	0.080	0.120	-	0.040	0.080	0.120
CH_4/g AA or Protein added	100.0	112.5	116.7	-	200	200	108.3

Figure 1.

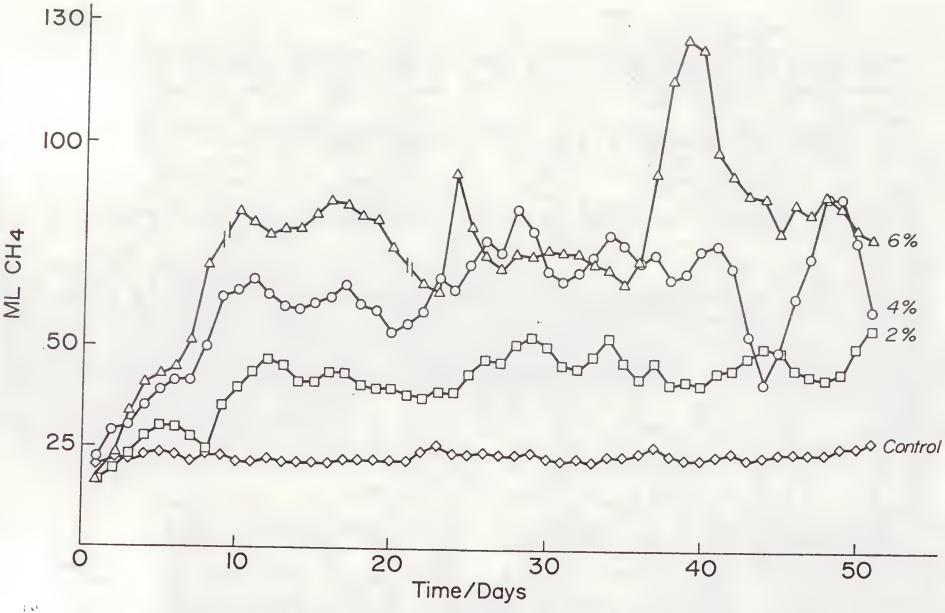


Figure 2

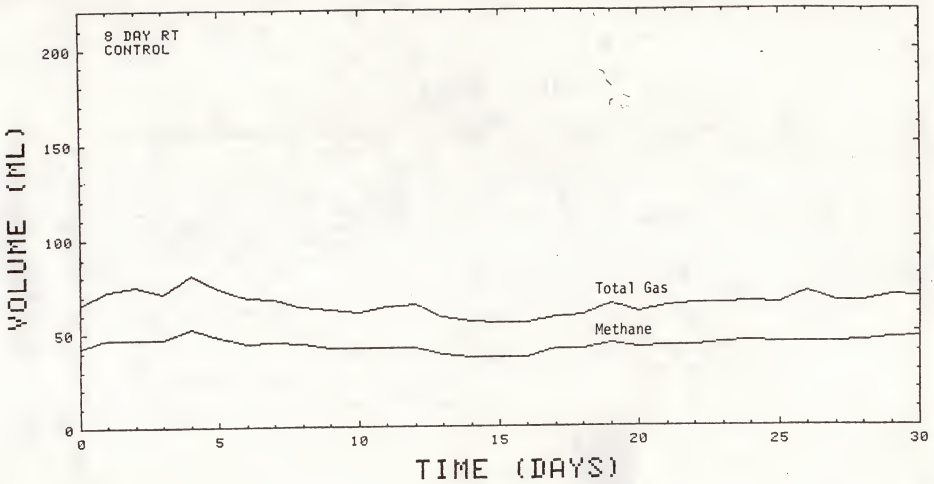

8 DAY RT
CONTROL
VOLUME (ML)
200
150
100
50
0
0
5
10
15
20
25
30
TIME (DAYS)
Total Gas
Methane

Figure 3

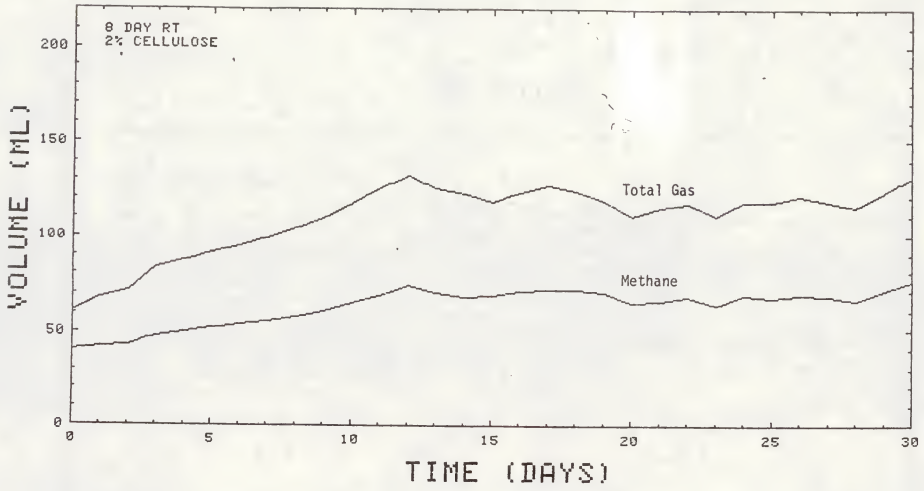

8 DAY RT
2% CELLULOSE
VOLUME (ML)
TIME (DAYS)
Total Gas
Methane
0
50
100
150
200
0
5
10
15
20
25
30

Figure 4

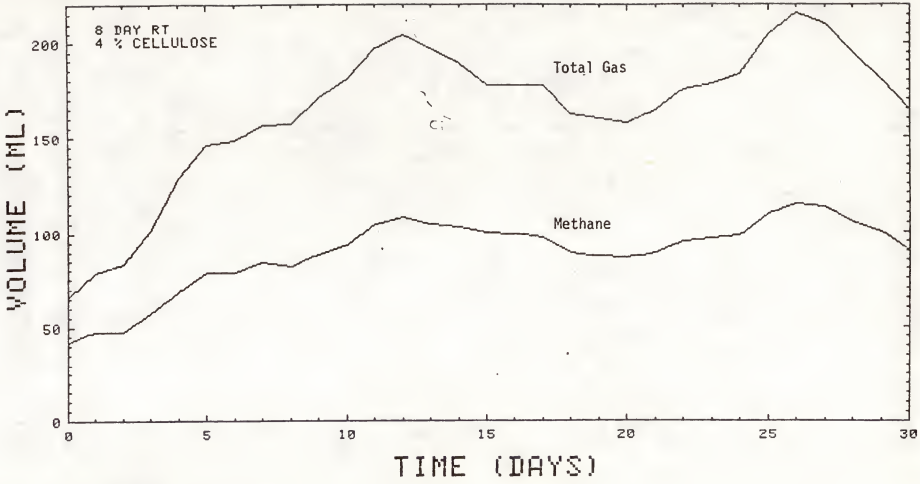

8 DAY RT
4 % CELLULOSE
Total Gas
Methane
VOLUME (ML)
TIME (DAYS)
0
50
100
150
200
0
5
10
15
20
25
30

Figure 5

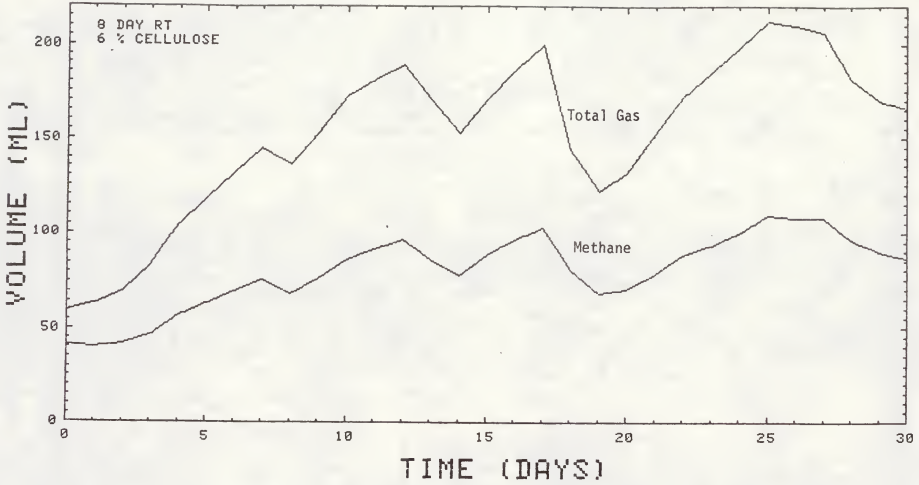

8 DAY RT
6 % CELLULOSE
VOLUME (ML)
TIME (DAYS)
Total Gas
Methane
0
50
100
150
200
0
5
10
15
20
25
30

Figure 6

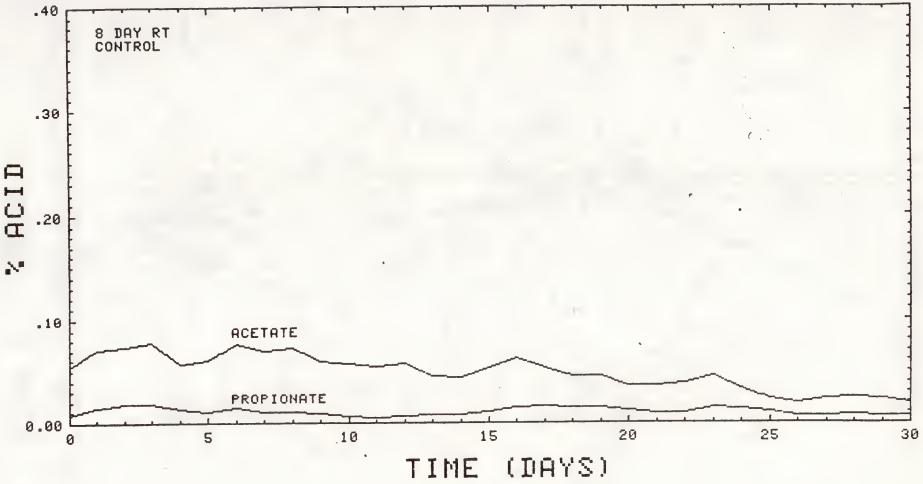

8 DAY RT
CONTROL
% ACID
.40
.30
.20
.10
0.00
ACETATE
PROPIONATE
0
5
10
15
20
25
30
TIME (DAYS)

Figure 7

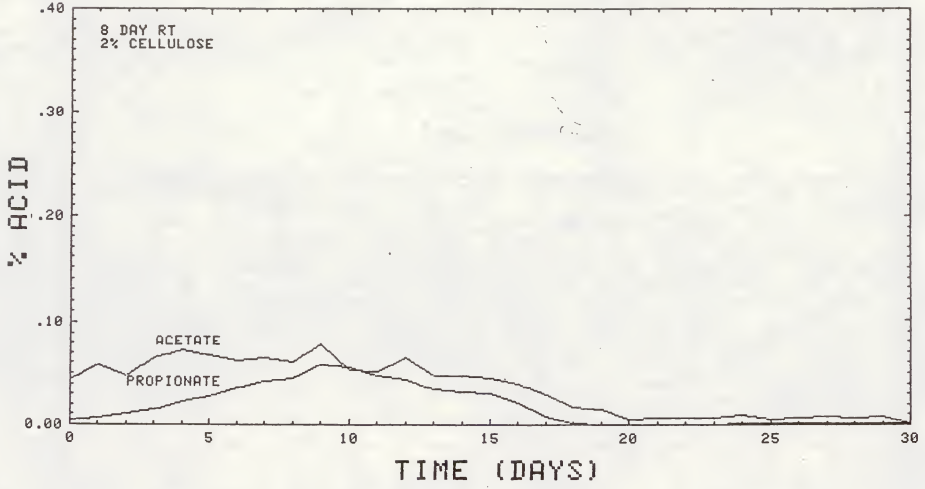

8 DAY RT
2% CELLULOSE
% ACID
.40
.30
.20
.10
0.00
ACETATE
PROPIONATE
0
5
10
15
20
25
30
TIME (DAYS)

Figure 8

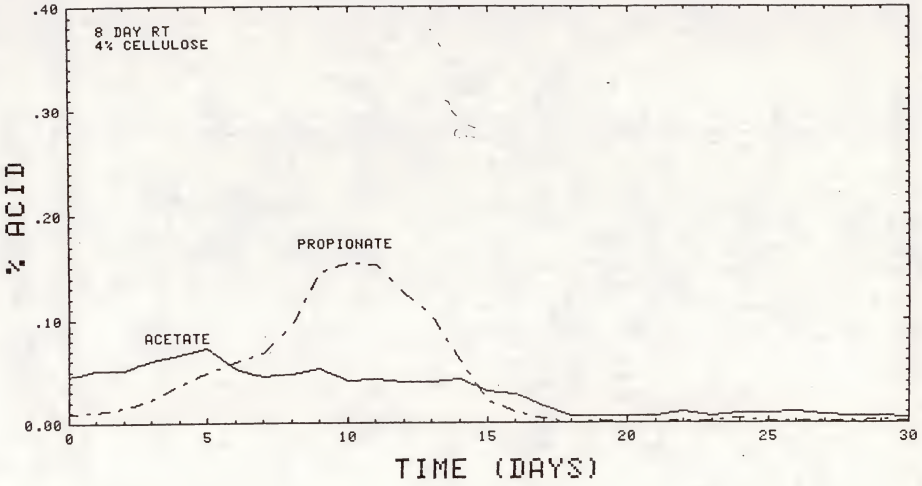

8 DAY RT
4% CELLULOSE
% ACID
.40
.30
.20
.10
0.00
PROPIONATE
ACETATE
0
5
10
15
20
25
30
TIME (DAYS)

Figure 9

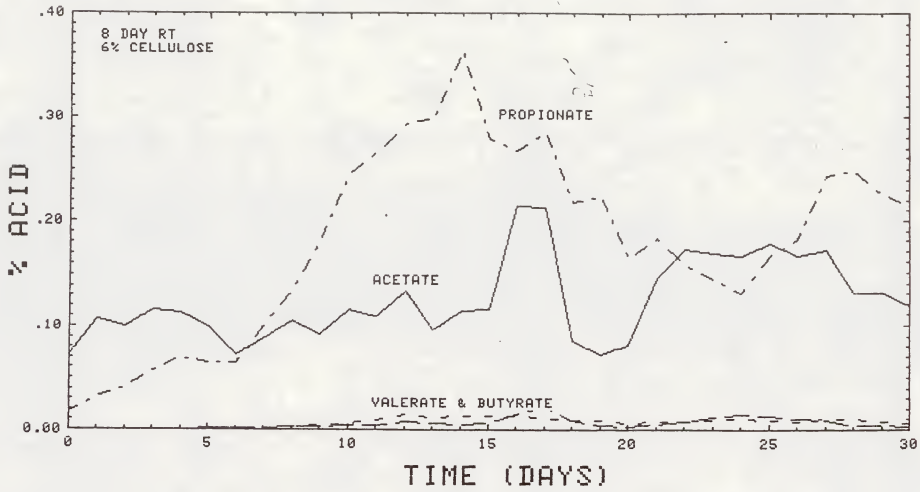

8 DAY RT
6% CELLULOSE
PROPIONATE
ACETATE
VALERATE & BUTYRATE
% ACID
.40
.30
.20
.10
0.00
0
5
10
15
20
25
30
TIME (DAYS)

Figure 10

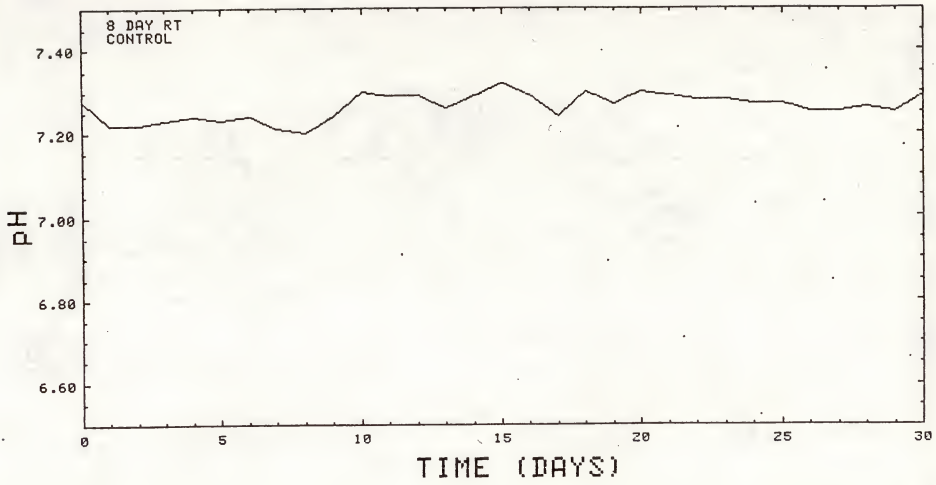

8 DAY RT
CONTROL
7.40
7.20
7.00
6.80
6.60
pH
0
5
10
15
20
25
30
TIME (DAYS)

Figure 11

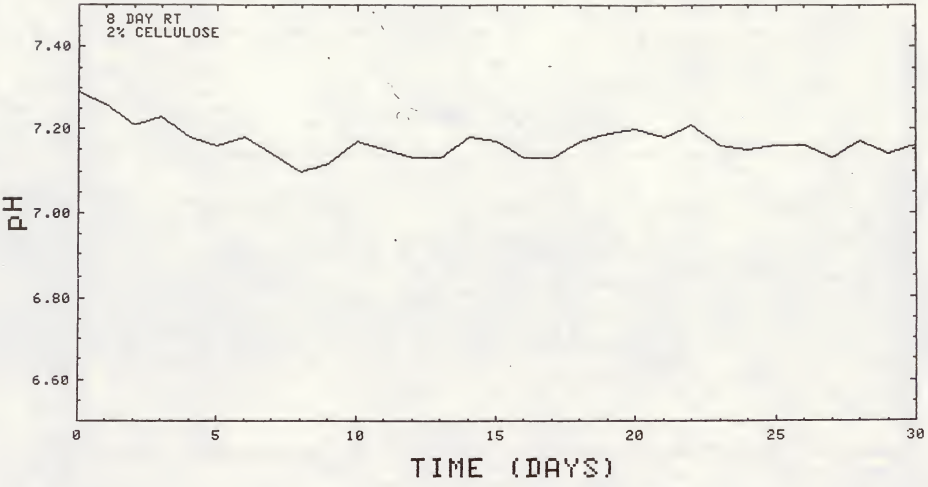

8 DAY RT
2% CELLULOSE
7.40
7.20
7.00
6.80
6.60
pH
0
5
10
15
20
25
30
TIME (DAYS)

Figure 12

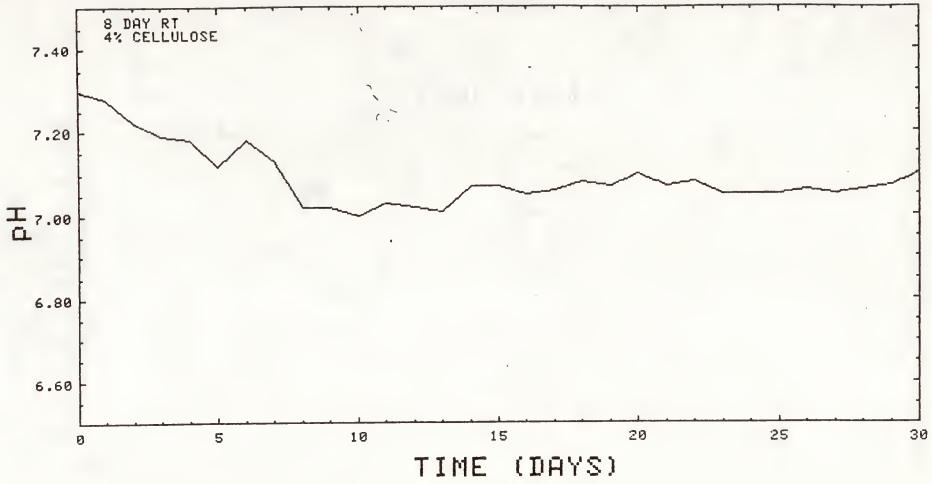

8 DAY RT
4% CELLULOSE
7.40
7.20
7.00
6.80
6.60
pH
0
5
10
15
20
25
30
TIME (DAYS)

Figure 13

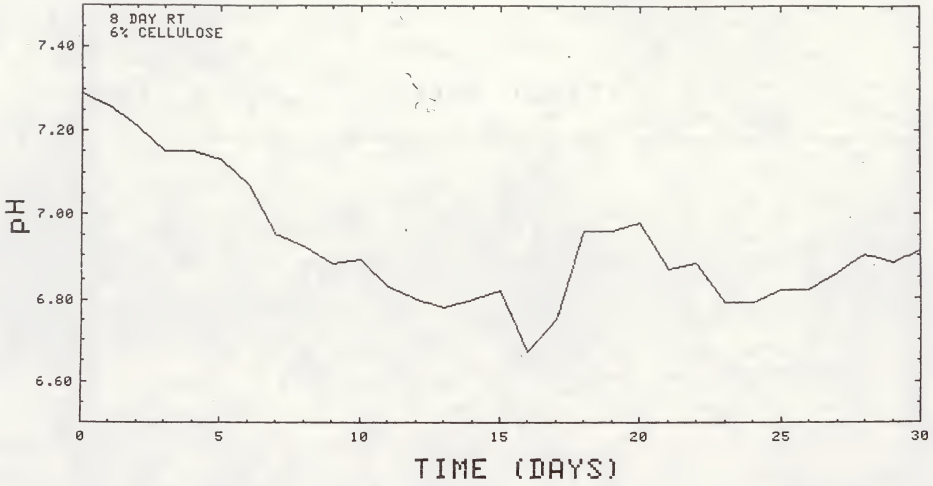

8 DAY RT
6% CELLULOSE
7.40
7.20
7.00
6.80
6.60
pH
0
5
10
15
20
25
30
TIME (DAYS)

Figure 14

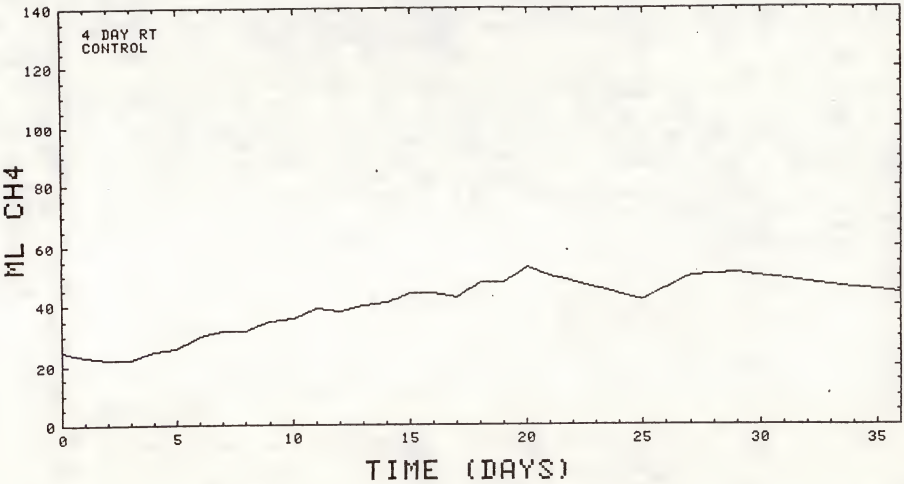

4 DAY RT
CONTROL
ML CH4
TIME (DAYS)
0
20
40
60
80
100
120
140
0
5
10
15
20
25
30
35

Figure 15

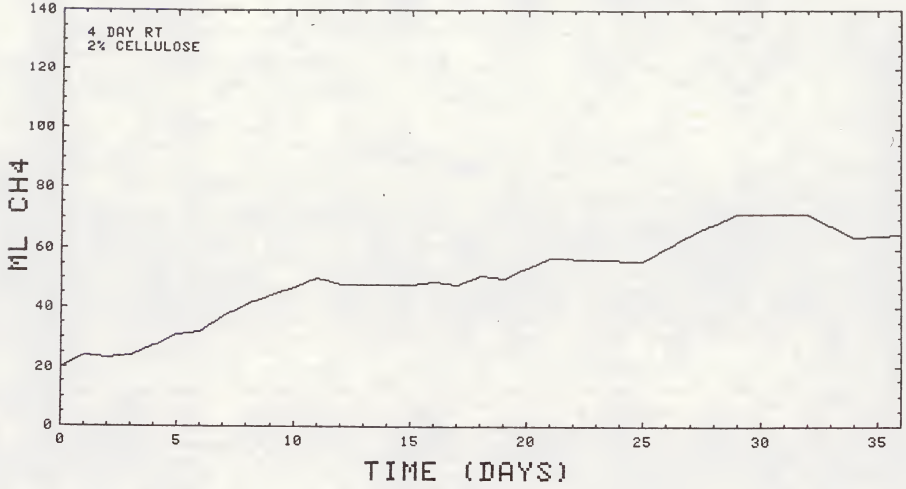

4 DAY RT
2% CELLULOSE
ML CH4
TIME (DAYS)
0
20
40
60
80
100
120
140
0
5
10
15
20
25
30
35

Figure 16

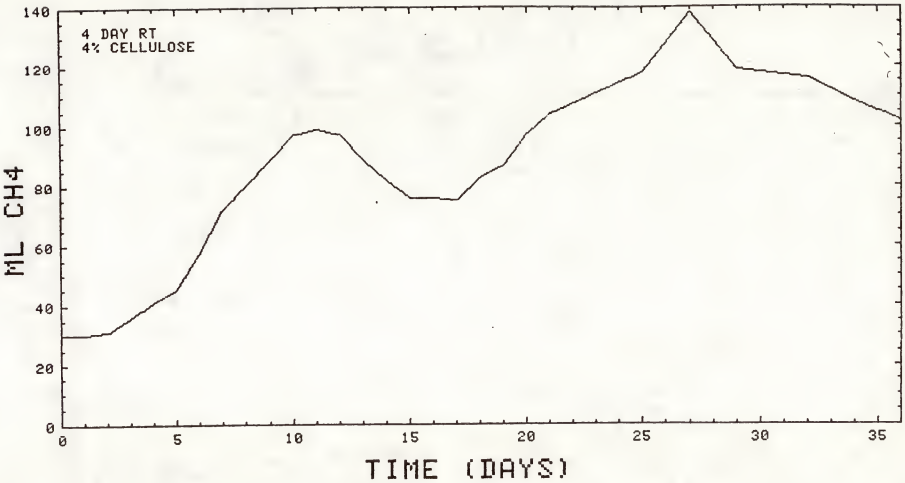

4 DAY RT
4% CELLULOSE
ML CH4
0
20
40
60
80
100
120
140
0
5
10
15
20
25
30
35
TIME (DAYS)

Figure 17

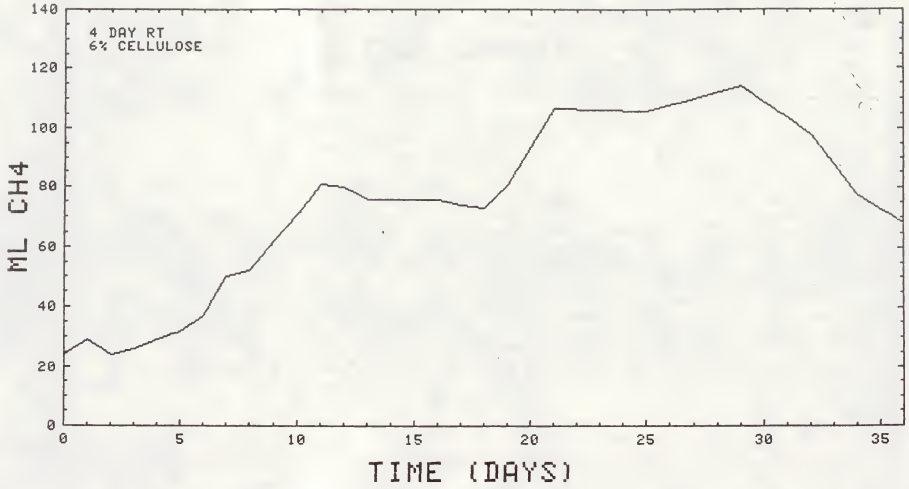

4 DAY RT
6% CELLULOSE
ML CH4
0
20
40
60
80
100
120
140
0
5
10
15
20
25
30
35
TIME (DAYS)

Figure 18

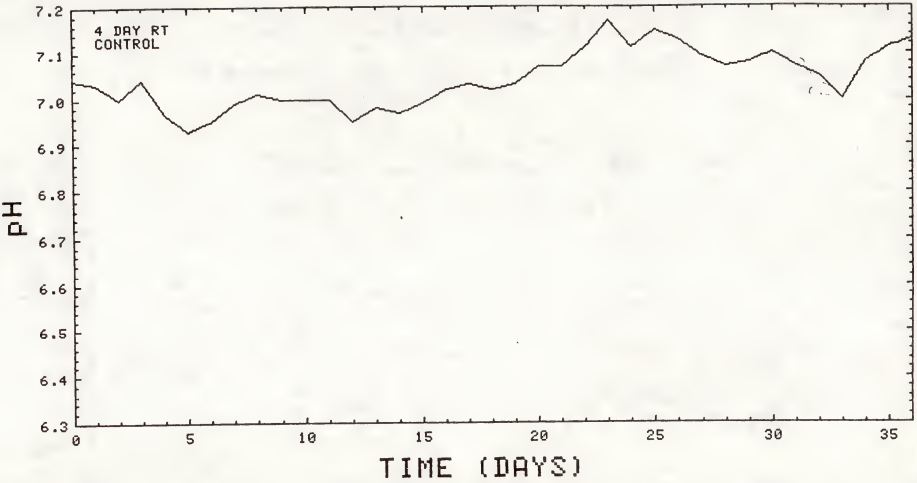

4 DAY RT
CONTROL
pH
7.2
7.1
7.0
6.9
6.8
6.7
6.6
6.5
6.4
6.3
0
5
10
15
20
25
30
35
TIME (DAYS)

Figure 19

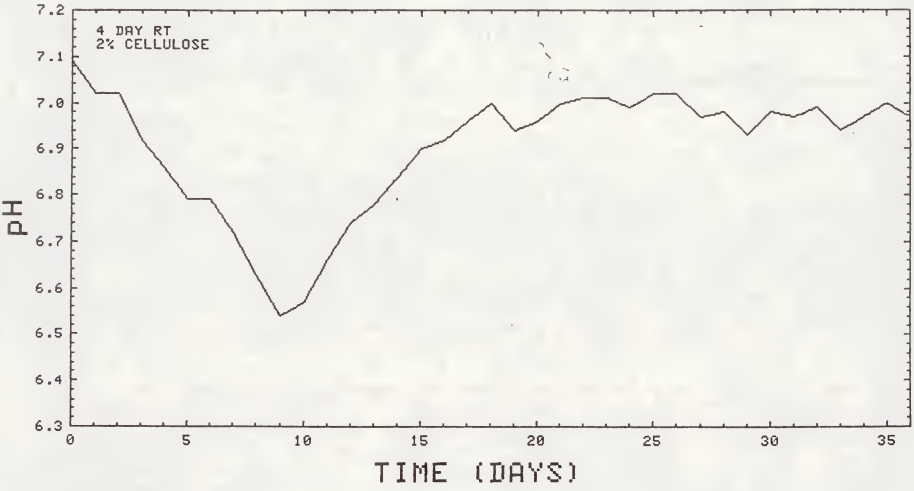

4 DAY RT
2% CELLULOSE
pH
7.2
7.1
7.0
6.9
6.8
6.7
6.6
6.5
6.4
6.3
0
5
10
15
20
25
30
35
TIME (DAYS)

Figure 20

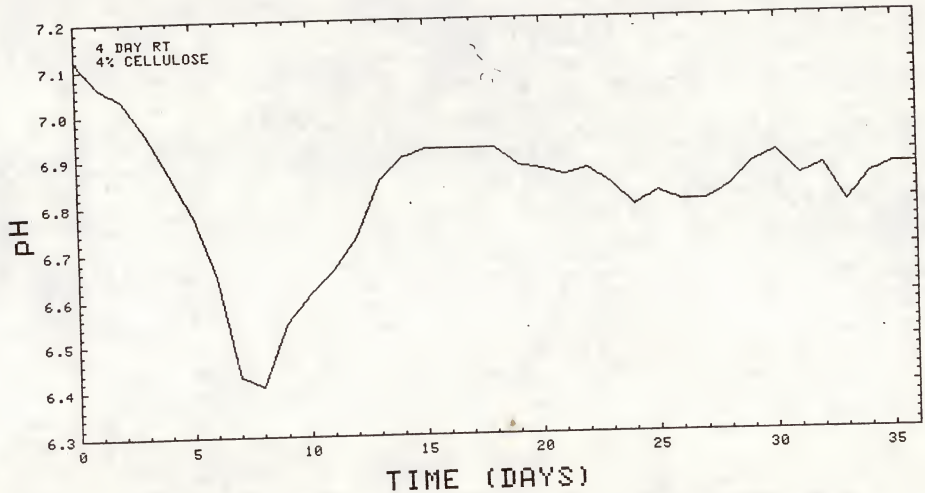

4 DAY RT
4% CELLULOSE
pH
7.2
7.1
7.0
6.9
6.8
6.7
6.6
6.5
6.4
6.3
0
5
10
15
20
25
30
35
TIME (DAYS)

Figure 21

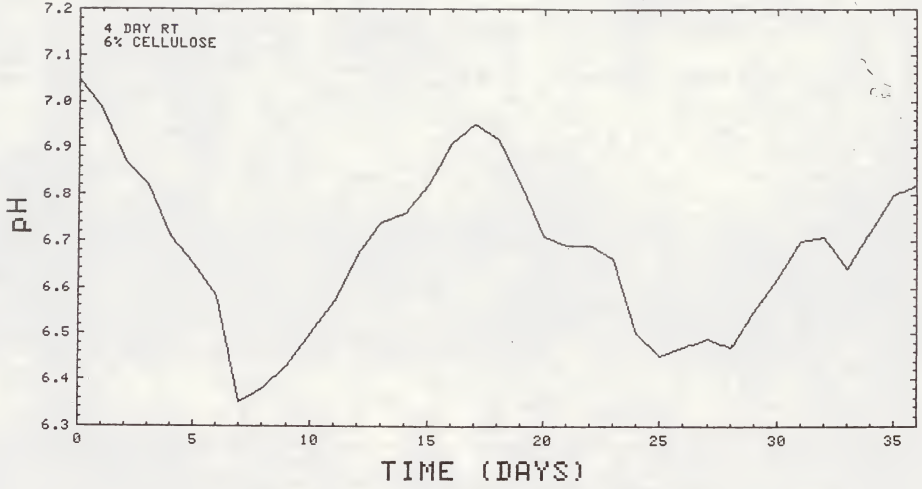

4 DAY RT
6% CELLULOSE
pH
7.2
7.1
7.0
6.9
6.8
6.7
6.6
6.5
6.4
6.3
0
5
10
15
20
25
30
35
TIME (DAYS)

Figure 22

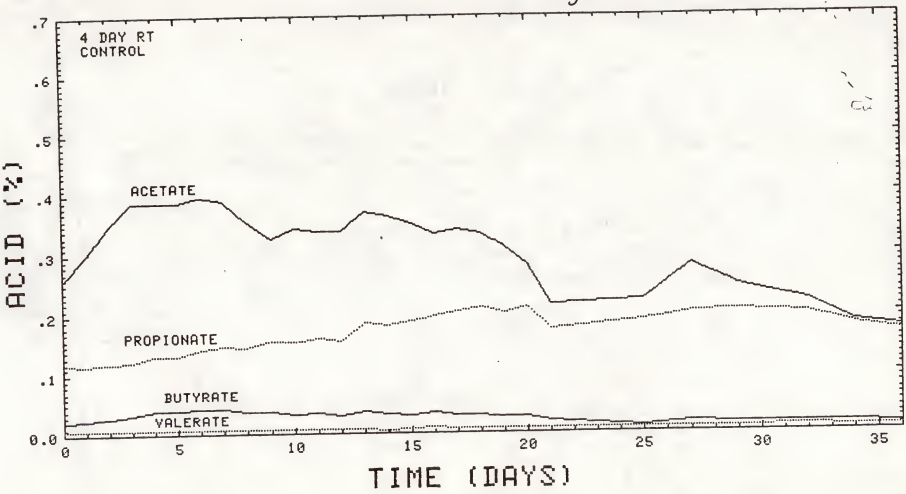

4 DAY RT
CONTROL
ACETATE
PROPIONATE
BUTYRATE
VALERATE
ACID (%)
.7
.6
.5
.4
.3
.2
.1
0.0
0
5
10
15
20
25
30
35
TIME (DAYS)

Figure 23

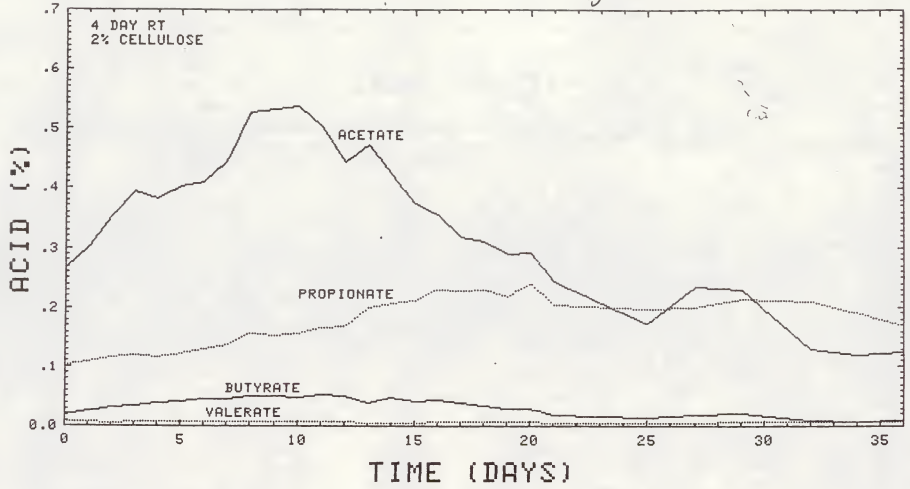

4 DAY RT
2% CELLULOSE
ACETATE
PROPIONATE
BUTYRATE
VALERATE
ACID (%)
.7
.6
.5
.4
.3
.2
.1
0.0
0
5
10
15
20
25
30
35
TIME (DAYS)

Figure 24

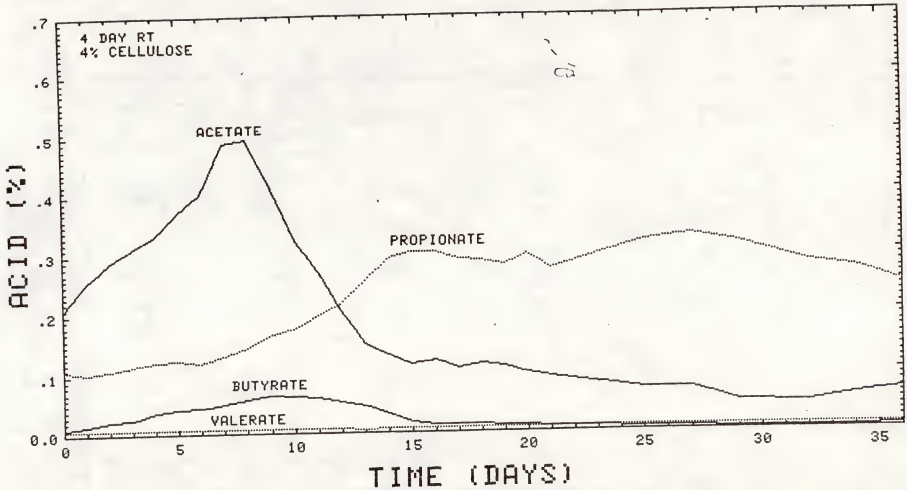
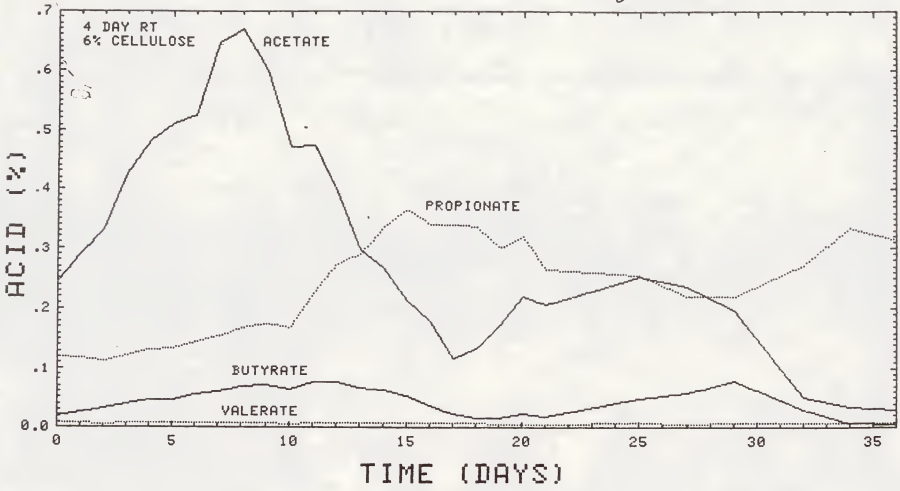

4 DAY RT
4% CELLULOSE
ACETATE
PROPIONATE
BUTYRATE
VALERATE
ACID (%)
TIME (DAYS)
.7
.6
.5
.4
.3
.2
.1
0.0
0
5
10
15
20
25
30
35

Figure 25


4 DAY RT
6% CELLULOSE
ACETATE
PROPIONATE
BUTYRATE
VALERATE
ACID (%)
.7
.6
.5
.4
.3
.2
.1
0.0
0
5
10
15
20
25
30
35
TIME (DAYS)

LEGENDS FOR FIGURES

Fig. 26 Methane production from manure and several glucose concentrations. The feed composition for these fermentations were 5% manure solids plus 1(o), 2(Δ), 3(□), 4(▽), 5(▲) and 6%(■) added glucose. Methane produced in the last 12 hours of a 24 hour period is shown.

Fig. 27 Acetate concentrations in fermenters that received 5% solids manure + 1, 2, 3, 4, 5 and 6% glucose.

Fig. 28 Propionate concentrations in fermenters that received 5% solids manure + 1, 2, 3, 4, 5 and 6% glucose.

Fig. 29 Methane produced in 24 hour periods from fermentations of 5% manure (⊙, plot C), 5% manure plus 2% glucose (○, plot 1), 5% manure, 2% glucose and 1% acetate (Δ, plot 2), 5% manure, 2% glucose, 2% HCO_3^- and H_2 (□, plot 3), 5% manure, 2% glucose, 1% acetate, 2% HCO_3^- and H_2 (▽, plot 4), 5% manure and 6% glucose (Δ, plot 5), 5% manure, 6% glucose and 1% acetate (■, plot 6), 5% manure, 6% glucose, 2% HCO_3^- and H_2 (●, plot 7), and 5% manure, 6% glucose, 1% acetate, 2% HCO_3^- and H_2 (▼, plot 8).

Fig. 30 Volatile acid profiles for fermentations as described for Fig. 5 for 6% glucose and 6% glucose plus methanogenic substrates. The ordinate label mM applies only to propionate.

Figure 24

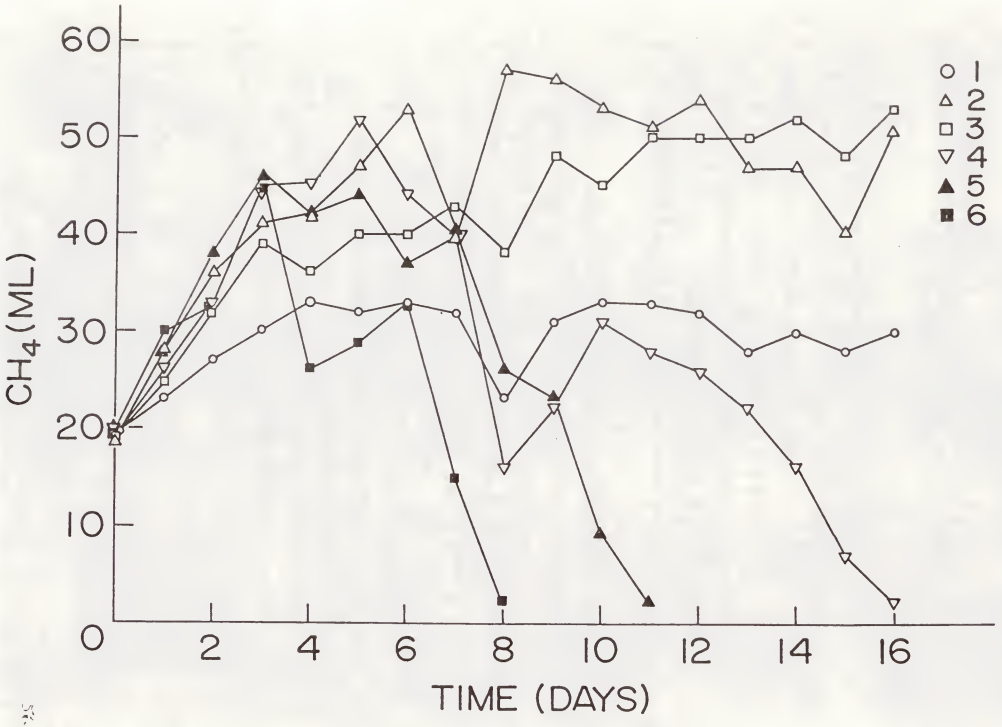


Figure 27

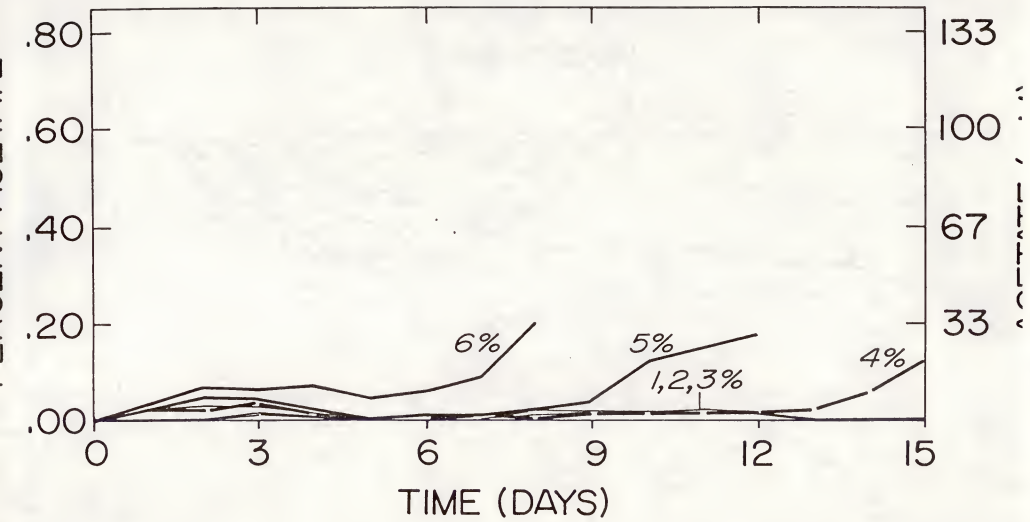


Figure 28

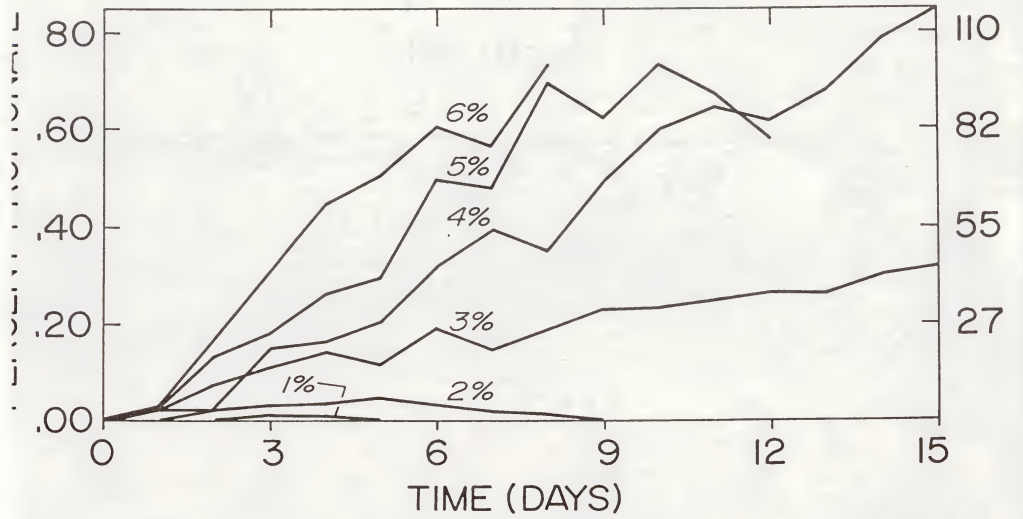


Figure 29

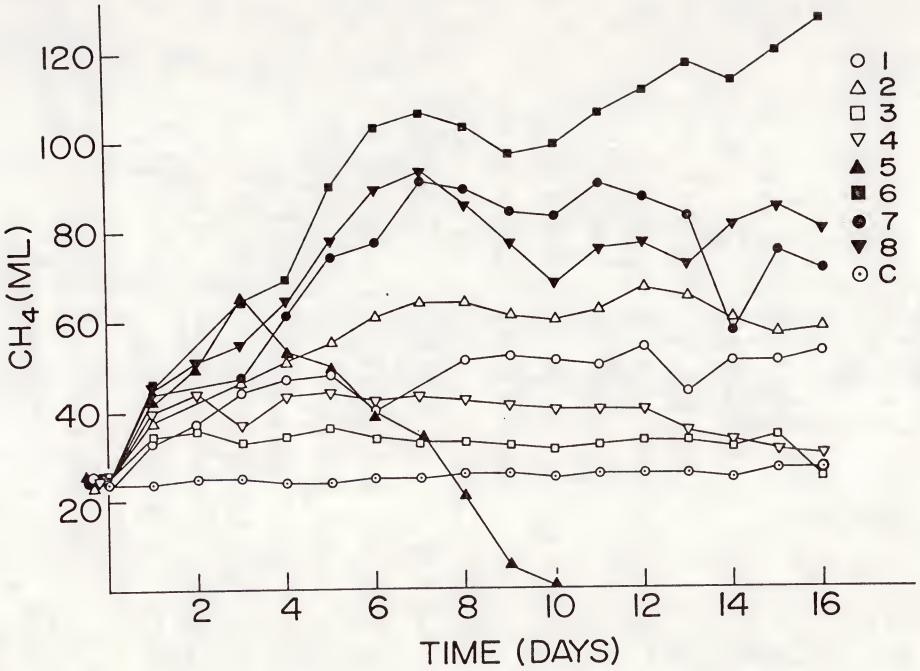


Figure 30

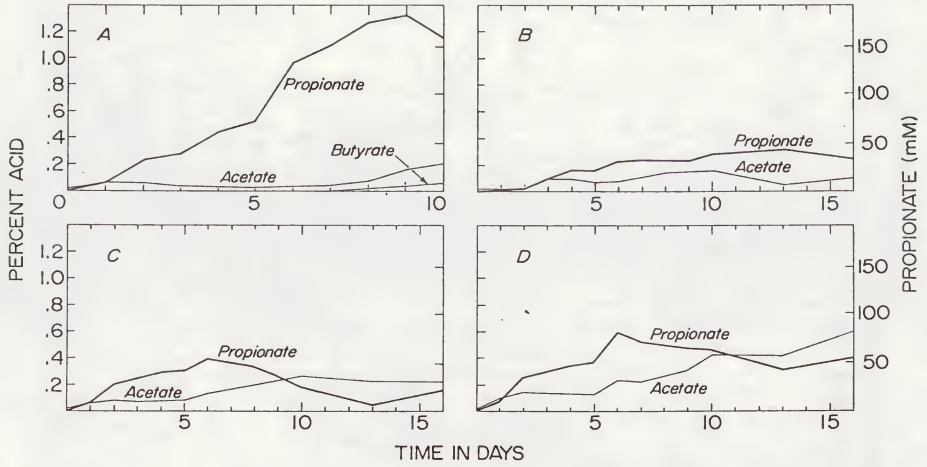

A
B
C
D
Propionate
Butyrate
Acetate
PERCENT ACID
PROPIONATE (mM)
TIME IN DAYS
0
5
10
15
.2
.4
.6
.8
1.0
1.2
50
100
150

Figure AA P-1

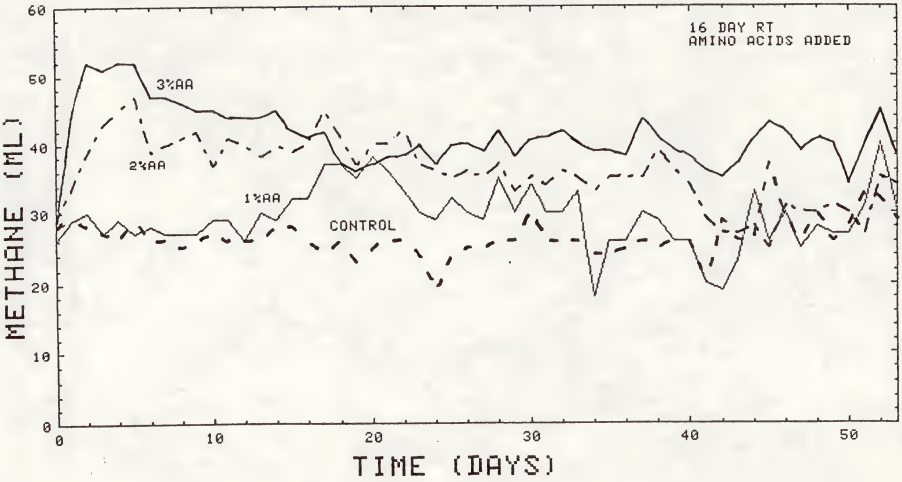
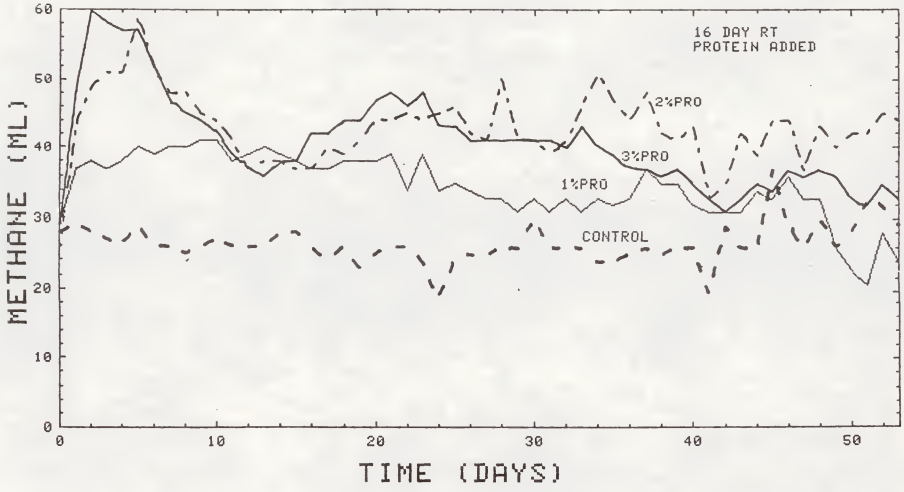

16 DAY RT
AMINO ACIDS ADDED
3%AA
2%AA
1%AA
CONTROL
METHANE (ML)
TIME (DAYS)
0
10
20
30
40
50
60

Figure AAP-2


16 DAY RT
PROTEIN ADDED
2%PRO
3%PRO
1%PRO
CONTROL
METHANE (ML)
TIME (DAYS)
0
10
20
30
40
50
60

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INTRODUCTION

The objectives of this proposal are reiterated here:

1. Evaluation of the failure process of digestors receiving high concentrations of nutrients and/or short retention times. The metabolic activity of the microbial flora will be monitored via analyses of gas production, volatile acids, ammonium ion and pH.

2. The optimum loading concentrations will be defined for maximum methane production, stable fermentation and reasonable volatile solids destruction, e.g. 33%.

3. Appropriate monitoring analyses that signal the on set of digestor failure will be established.

SUMMARY OF ACCOMPLISHMENTS

1. ^{13}C-Nuclear Magnetic Resonance Spectroscopy.

We reported in our first quarterly report the advent of the application of carbon-13 nuclear magnetic resonance spectroscopy to the study of metabolic interactions in anaerobic digestors. Since that report we have submitted copies of a recent publication as evidence of accomplishment in this area. The publication serves best as a report of the findings in this study of the utility of ^{13}C-NMR spectroscopy. The technique has proven to be a valuable tool for investigations of metabolic interactions in the complex ecosystem of anaerobic digestion. The support of this grant has thus provided researchers with an important investigative tool. We are receiving requests for reprints from all over the world regarding this work.

2. Metabolic Interactions Among Microbial Species.

In the second and third quarterly reports we described studies on metabolic imbalance that can arise from high nutrient loading for digestors. These studies have endured over several years and clarification of the complex interplay has been difficult. However, the impact of high nutrient loads on metabolic and microbial population changes has been greatly clarified within the past year. We do not intend to reiterate the details of these studies in this report since these details have been reported previously. We present here a short summary of our findings.

The fermentation of high concentrations of soluble energy in the form of glucose led to imbalance in the production and utilization of organic acids within the digestors. Large amounts of lactate were produced from the glucose and relatively small amounts of acetate were produced. The lactate was subsequently converted to propionate and acetate. The larger portion of the lactate was converted to propionate. The events that lead to this result are population enhancement of fermentative bacteria which produce lactate from glucose and those which can reduce lactate to propionate. The effects of this result are a reduced supply of acetate to the methanogenic bacteria which in turn leads to reduced methane production and buffering capacity in the form of bicarbonate. Also the overproduction of propionate which must be metabolized by the acetogenic bacteria leads to a decrease in digestor pH. The propionate is recalcitrant and provides an accumulative detrimental effect on the fermentation

process which leads to digestor failure if it is not metabolized or if some buffering is not supplied. Supportive evidence for this view was obtained from fermentations of high concentrations of glucose with added acetate. The addition of acetate provided stable and productive fermentations. Parallel experiments on fermentation of high concentrations of glucose with added bicarbonate also provided stable and productive fermentations. Thus, the ultimate detrimental event was decrease in pH to the point where propionate and acetate utilization was impaired. The buffering is typically provided by the metabolism of acetate by the methanogenic bacteria. In the case of high concentrations of glucose alone, little acetate was produced and thus inadequate buffering resulted. In fermentations which were stablized by added acetate the buffering that resulted from metabolism of the acetate provided adequate time for enrichment of the slow growing acetogenic bacteria. This then allowed for increased utilization of the propionate produced from glucose via lactate. These fermentations, therefore, were able to completely utilize the propionate and reduced the concentration levels to trace amounts.

These studies provided a description of metabolic and population shifts upon receiving high concentrations of glucose but they did not provide the reason that so much propionate was produced. Previous studies by other investigators has clearly shown the importance of interspecies hydrogen transfer. Thus, one could expect that propionate was produced as an alternative electron sink other than hydrogen ions (H^+). The production of molecular hydrogen (H_2) from H^+ is known to be particularly sensitive to the concentration of H_2 in the digestor because of thermodynamic considerations. Our studies on H_2 concentrations in digestors following a feeding of high concentrations of glucose indicate that H_2 concentration does not inhibit its own formation and thus cause an increase in propionate production. Further, from studies with ^{13}C-NMR it is clear that the H_2 concentration is not responsible for inhibiting propionate utilization. Thus, it seems most likely that the production of lactate, and subsequently propionate, was simply a response to a need for oxidizing power (NAD+). The metabolic events have been described which follow feeding of high concentrations of immediately available energy, e.g. glucose. These studies suggest that caution should be excercised when contemplating the inclusion of such wastes in anaerobic digestors or in considering pretreatments of wastes by acid hydrolysis to circumvent the rate limitations of hydrolysis. Further, these studies indicated that anaerobic digestors may be acclimated to fermentations of high concentrations of nutrients.

In the remaining period we have attempted to show that acclimation of acetogenic bacteria populations is possible and secondly that a result of this enrichment would be a sparing effect against failure as witnessed when high concentrations of glucose were fermented. In our last quarterly reports we had some encouraging results but had not been able to avert failure. In recent attempts we have been able to acclimate digestors to high concentrations of propionate (10 mm). This may be assumed to indicate an increase in the acetogenic population. These digestors were then subjected to feeding of high concentrations of glucose. The concentration being the same as that which previously produced failure (20 mM). These digestors are stable and very productive. This indicates that with improved ability within a digestor to metabolize propionate comes added stability and high productivity. Overall, these studies then suggest that in the boundary conditions of a high performance digestor that the catabolism of propionate or other higher molecular weight organic acids becomes a critically important factor.

The objectives outlined for this proposal have been successfully fulfilled.

1. The failure process has been described.
2. The optimum loading concentrations were found to be waste mixtures containing approximately 40% cellulosic material as the fraction of volatile solids. Solids destruction achieved was near 50%. These results have been submitted in previous reports.
3. The earliest signal of the on set of failure on an imbalance in the fermentation process is an increase in volatile acids, such as propionate, butyrate or isobutyrate and in some instances isovalerate. These acids increase in concentration prior to a notable decrease in pH.

We gratefully acknowledge the financial support we have received and the cooperation shown us by the staff in the Energy Bureau section of the Montana Department of Natural Resources and Conservation.

BUDGET REPORT

October 15, 1981

Salaries	$20,358.11		$23,000.00
Benefits	3,942.22		4,025.00
Repair & Maint.	598.19		
Travel	615.71		600.00
Computer	300.00		
Supplies	1,451.33		2,000.00
Subcontract	308.26		
Administration	7,290.10		8,107.00
	$34,863.92		$37,732.00
		Balance	$ 2,868.08

msu

DEPARTMENT OF CHEMISTRY

COLLEGE OF LETTERS & SCIENCE

MONTANA STATE UNIVERSITY, BOZEMAN 59717

QUARTERLY PROGRESS REPORTS

for the

Energy Bureau of the

MONTANA DEPARTMENT OF NATURAL RESOURCES AND CONSERVATION

on Grant No. RAE 404-801

TITLE: Factors Affecting Bioconversion and Methanogenesis in Anaerobic Digestors

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PERIOD:

November 1980-January 1981

February 1981-April 1981

May 1981 - August 1981

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PERIOD: November 1980-January 1981

The studies during this period were a continuation of previous studies to identify processes or factors involved in destabilization of anaerobic fermentation. Earlier investigations have shown that enrichments of fermentative bacteria occurred with feed stock containing high concentrations of glucose. The addition of acetate to these feed stocks stabilized the fermentation. Evidence was obtained that indicated that high concentrations of propionate produced in the earlier fermentations led to instability and that the inclusion of acetate allowed enrichments of methanogenic bacteria. The latter enrichment provided stability. We raised the question of, can digestors on energy rich feed stock also be stabilized by enrichment of the acetogenic class of bacteria? Thus we established fermentations which included high concentrations of glucose (0.333M) and added 0.640M propionate and also the same amount of glucose and propionate but also 0.169M acetate which had provided stability before. Digestors were maintained at $37^{o}C$ with a 16 day retention time for ca. 36 days. Failure of these fermentations occurred at this time. The experiment was repeated with the same result. Analyses of the volatile acids indicated that accumulation of many volatile acids occurred, which included propionate, valerate, butyrate and isovalerate. This treatment was, thus, not successful at enriching for acetogenic bacteria. The time required for these experiments and the analyses was approximately 3 months.

PERIOD: February - April 1981

A second try at enriching for acetogenic bacteria was made in this period. The fermentations established were the same except the propionate concentration was decreased to 0.320M rather than 0.640M. These fermentations were also attempted twice. These fermentations lasted much longer (viz. 85-92 days) These fermentations did fail after this time period. The fact that these fermentations lasted so much longer we felt was encouraging as to the possibility of stabilizing fermentation via enriching for acetogenic bacteria. The volatile acid analyses again indicated accumulation of volatile acids and ethanol had occurred. The experiment will be repeated at 0.160M propionate. These studies required 4 months.

PERIOD: May 1981 - August 1981

In April the question of how added acetate was able to provide stabilization was raised. Was stabilization due to a buffering effect from the metabolism of acetate by the methanogenic bacteria? was the specific question. To put this question to test, fermentations were established which recieved feed stock containing 0.333M glucose and either 0.169M potassium acetate or 0.169M potassium bicarbonate. The fermentation of potassium bicarbonate would essentially be just buffering the system. The results of these fermentations are shown in Fig. 1, 2 and 3. As can be seen there were only minor differences between fermentation of acetate as opposed to fermentation of bicarbonate. Thus we conclude that the stabilizing effect of adding acetate was in part due to a buffering effect. The time period required for these experiments and analyses was 90 days.

Fig 1

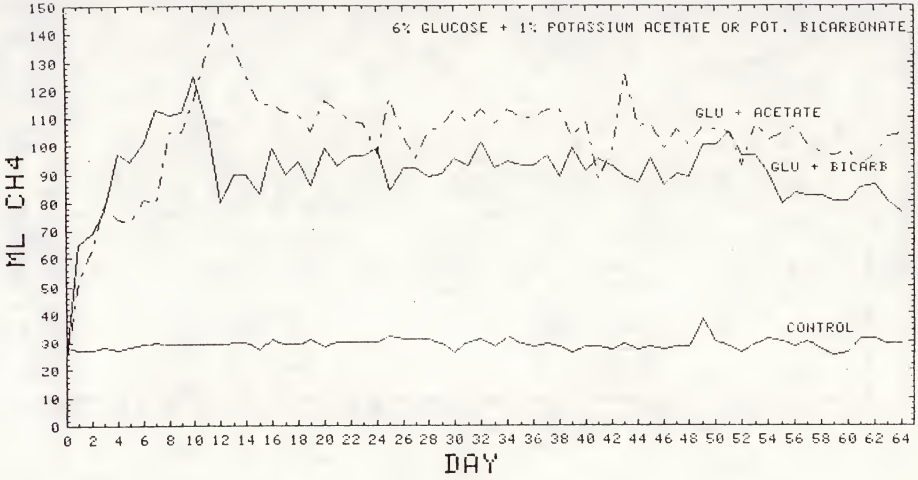

6% GLUCOSE + 1% POTASSIUM ACETATE OR POT. BICARBONATE
GLU + ACETATE
GLU + BICARB
CONTROL
ML CH4
DAY
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64

Fig 2

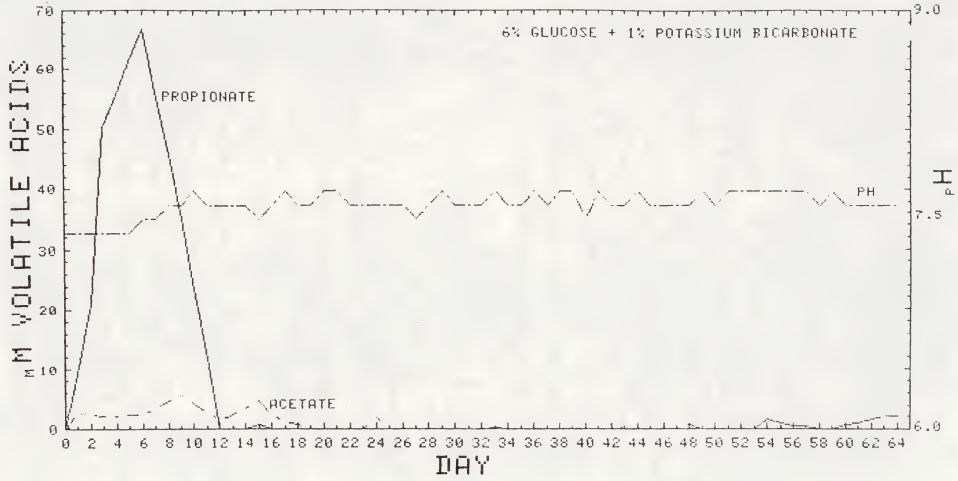
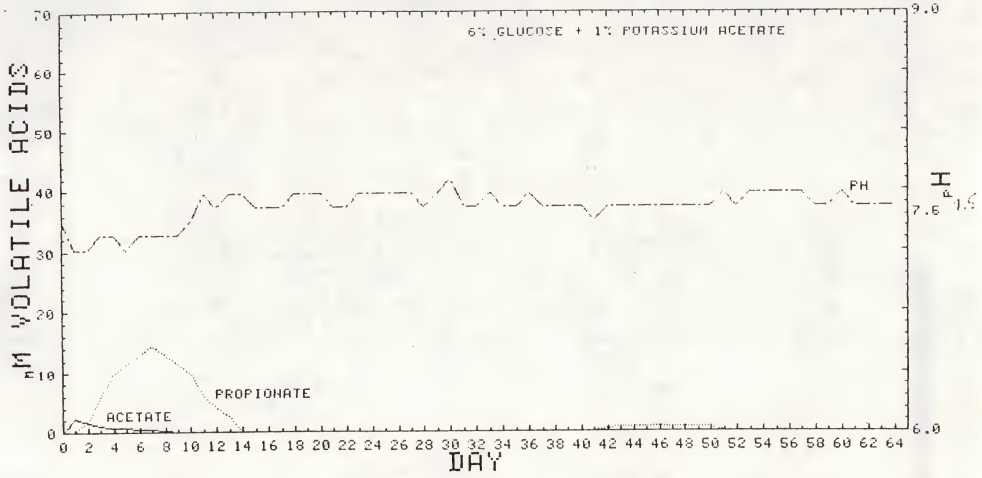

6% GLUCOSE + 1% POTASSIUM BICARBONATE
PROPIONATE
ACETATE
PH
mM VOLATILE ACIDS
pH
DAY
0 10 20 30 40 50 60 70
6.0 7.5 9.0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64

Fig 3


6% GLUCOSE + 1% POTASSIUM ACETATE
mM VOLATILE ACIDS
pH
PH
PROPIONATE
ACETATE
DAY
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64
0 10 20 30 40 50 60 70
6.0 7.6 9.0

[redacted]

Factors Affecting Bioconversion and Methanogenesis in Anaerobic Digestion

Grant No. RAE 404-801

Time Period: Nov 1980-Jan 1981

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INTRODUCTION

For this report I am providing documentation of our progress in the form of manuscripts submitted for publication which describe the results of studies aimed at the proposed objectives. The objectives of this study were:

1/ Evaluation of the failure process of digesters receiving high concentrations of nutrients or operated on short retention times. The metabolic activity of the microflora will be monitored via analyses of gas production, volatile acids, ammonium ion and pH. 2/ The optimum loading concentrations will be defined for maximum methane production, stable fermentation and reasonable volatile solids destruction, eg. 33% or better. 3/ Appropriate monitoring analyses that signal the onset of digester failure will be established.

The manuscripts entitled; 1/ Effects of Glucose and Acetate Additions on Anaerobic Digester Performance; 2/ Hydrogen Accumulation in Anaerobic Digesters and 3/ Application of ^{13}C-NMR to Observing Metabolic Interactions in Anaerobic Digesters have addressed objectives 1 and 3. The manuscript entitled; Anaerobic Digestion of Cellulose-Manure Mixtures, addressed objective 2.

SUMMARY AND SIGNIFICANCE OF RESULTS

The studies on the affects of adding acetate along with the high energy load in the form of glucose provided several important findings:

1. Pretreatments such as hydrolysis to avert the rate limitations of converting cellulose and hemicellulose to sugars will not be advantageous, rather, this would be detrimental for methane production.
2. Under operating conditions of readily available energy in high concentrations, the metabolism of glucose is shunted toward propionate rather than acetate. This results in a negative effect on methane production rates and leads to digester instability.

3. Under these conditions the methanogenic bacteria cannot keep pace with the fermentative bacteria due to the lack of acetate rather than an over supply of acetate.

The studies on hydrogen accumulation in digesters pointed out one major point. The current concept is that propionate and other volatile acids accumulate in digesters because hydrogen inhibits its own formation. This requires the fermentative bacteria to seek an alternative electron sink, ie. propionate rather than hydrogen. The study we performed showed that propionate and hydrogen accumulated simultaneously. We point out that this could not be possible if hydrogen inhibition of its own formation caused propionate production. Propionate would accumulate following a peak in hydrogen concentration if the above were true. The results we present indicate that energy metabolism in the fermentative class exceeds their own capability to dispose of electrons. Not that methanogens utilize hydrogen too slowly. This implies that enrichment for methanogens prior to high energy loading may not be as advantageous as thought under the previous concept.

The study which showed that NMR spectroscopy could be applied to the study of metabolism in anaerobic digesters has provided some interesting insight to the studies above. We have been able to monitor the metabolic products of glucose metabolism very readily. The previous studies did not indicate the large production of lactic acid from glucose because the lactic acid produced is converted to propionic acid (propionate). The NMR study showed the time sequence of metabolic events and thus we now know that the propionate produced in the previous experiments was primarily produced from lactate. This study confirms our hypothesis that fermentative bacteria need an alternative electron sink for disposal of electrons. The lactate production occurs immediately upon feeding glucose. Thus, hydrogen inhibition of its own formation is not the cause of propionate accumulation.

Determination of Sulfide by Flow Injection Analysis with Gaseous Diffusion Separation and Amperometric Detection

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Abstract

Manure-cellulose mixtures containing 0-59% of the volatile solids as added cellulose were digested in laboratory scale digesters. Methane yields, volatile acid content and pH were determined for digesters operated at 16, 8 and 4 day retention times (RT). Methane yield and % conversion of volatile solids indicated that manure-cellulose mixtures comprised of ca. 40% volatile solids as added cellulose provided the optimum mixture. Volatile acid and pH profiles from 4d RT experiments indicated these digesters did not attain steady state even after 9 RT. Statistical analysis of methane yield from digesters receiving 59% volatile solids as added cellulose on 16d RT indicated these digestions had not reached a true steady state after 3 RT. These observations suggest that digesters receiving high energy loads may not attain a true steady state.

Introduction

Many intensified livestock operations already provide treatment for the wastes produced to avoid or minimize air or water pollution. A promising treatment of wastes is anaerobic digestion with the added attraction of fuel production. Further increases in energy costs is expected to enhance the start up of on-farm anaerobic digesters. Anaerobic digestion can thus serve two important purposes, waste treatment and fuel production. According to Fraser (1) methane yields from manures can be improved by addition of cellulosic wastes. Several studies of anaerobic digestion of mixtures of manure and plant matter have been conducted (2, 3, 6, 7). All of these studies showed greater methane production from digestion of manure and cellulosic mixtures than from an equivalent amount of manure. The studies of Hills (2) and Hills and Roberts (3) indicated the importance of the C:N ratio in optimizing methane production. The study presented here was undertaken primarily to assess the optimum mixture of cattle manure and cellulose which provided good methane yields and reasonable destruction of volatile solids (VS). We feel it is important to stress the dual purpose of waste treatment and fuel production. This study assessed the methane yields per unit V.S. destroyed for several loading concentrations at three retention times (RT). This study was also concerned with possible effects of the addition of iron on the oscillatory methane production.

This study showed that mixtures of cellulose and manure in the range of 41 to 54% of the volatile solids as added cellulose yielded the optimum efficiency regardless of the retention time. The addition of iron did not alter the methane production profile or the

conversion efficiency.

Material and Methods

Manure and inoculum

The history, collection and handling of the dairy manure has been described previously (7). The composition of the manure given in Table 1 was determined by reported procedures (7). Inoculum was obtained from a 4 l fermenter maintained continuously for over two years on 5% manure solids at 37° with a 16d RT. The fermenters used in this study are illustrated in Fig. 1. These 100 ml fermenters were filled with a working volume of 64 ml inoculum and maintained in a 37° waterbath. Total gas production varied for the first 24 hrs. Within 3 to 9 days total gas production from each fermenter was within ± 5 ml of the mean gas production from the fermenters. Experiments were not initiated until stable conditions were established, as evidenced by pH and gas production.

Fermenter feeding

The fermenters were fed daily on a 16, 8 or 4d RT. They received 5% total solids manure plus base-treated cellulose powder in quantities described for each experiment. Preliminary experiments showed no significant differences in gas production between constant swirling of digesters and swirling only before and after feeding. Therefore the fermenters were swirled for 15 sec immediately before and after feeding. The performance and mechanical reliability of these fermenters is apparent from the graph of the control fermenters in Fig. 2. The fermenters used in the 8 and 4d RT experiments were conditioned on 5% manure solids for at least 3 retention times before initiating the experiment.

Assays

Gas volumes were determined by water displacement. Head space gas composition was determined by gas chromatography as previously described (7). Gas chromatographic analysis of volatile acids and determination of digest pH have been described (7).

Chemicals

Cellulose powder (Whatman CF 11) was place in 0.1 N NaOH at room temperature for one hour, then washed to neutral pH with water. The powder was then dried overnight at 75°, and used as needed. Other chemicals used were reagent grade.

Statistical Treatment of Data

Calculations of average daily methane yields were performed by linear regression. The conversion efficiencies were based on these values. Additional statistical analyses demonstrated that a second order autoregressive model adequately fit the daily methane yields.

Iron Additions to Feed

Iron was added to the feeds as $FeCl_2$ (0.04 g daily) for fermentations of 0, 2, 4 and 6% cellulose added solids. This provided a fermenter concentration of 5 mM, that noted by Hoban and van den Berg (4) as optimum for acetate conversion to methane.

Results

These experiments were primarily designed to determine the efficiency of cellulose utilization at several cellulose concentrations. The efficiency of utilization was also tested at 3 different retention times. A secondary objective, the effect of the addition of iron on the efficiency of conversion, was tested.

Methane production from cellulose additions at 16 d RT

Seven fermenters received daily additions of 5% manure solids and sufficient added cellulose to give concentrations of cellulose in the feed as 0, 1, 2, 3, 4, 5 and 6%. The total solids were 5, 6, 7, 8, 9, 10 and 11% solids in the feed. Experiments were conducted for 56 days. The methane production from 0, 2, 4 and 6% added cellulose is shown in Fig. 2A. Methane production from 1, 3 and 5% cellulose additions (not included in Fig. 2A) were intermediate between 0-2%, 2-4% and 4-6% respectively. The daily methane yields were irregular. The values oscillated throughout the experiment after an initial increase which occurred in the first 10-15 days. It was apparent that the amplitude of the oscillations was directly proportional to the loading concentration of cellulose.

Methane production from cellulose additions at 8d and 4d RT

In these experiments four fermenters at each RT were fed twice and four times the amount given for 0, 2, 4 and 6% added cellulose in the 16d RT experiment for 8d and 4d RT respectively. The methane production for 8d and 4d RT is shown in Figs. 2B and 2C, respectively. In general the daily methane yields behaved similarly (oscillatory) to the 16d RT. The 4% cellulose addition was more productive than 6% overall. The initial acclimation period in the 8d RT fermenters appeared to be similar to the 16d RT fermenters, i.e. approximately 13 days, whereas, the 4d RT fermenters did not appear to acclimate within the 36 day period.

Conversion efficiencies

A comparison of the conversion efficiencies and methane yields per unit weight of added cellulose and volatile solids (V.S.) is given in

Table 2. The percent conversion of V.S. and the methane yields per g of cellulose and per g of V.S. for the 16d RT were virtually the same for 3, 4 and 5% added cellulose, i.e. where added cellulose comprised 41 to 54% of the V.S. added. The 8d and 4d experiments indicated the 4% added cellulose (49% of added V.S.) was the optimum mixture.

Volatile acid concentration and pH

The volatile acid concentrations in fermenters on the 16d RT never reached very high concentrations, i.e. the maximum concentration attained was 6 mM and that was short lived. The change in pH throughout the experiment was minor. The initial pH values were 7.7 ± 0.1 and the final pH range was 7.5 to 7.2. The added cellulose concentration and pH were inversely related.

Accumulation of acetate and propionate was noted in all digesters on an 8d RT. The fermentation of manure only had an acetate concentration of 10 mM on the first day of the experimental period, attained a maximum concentration of 13 mM and then slowly declined to 3 mM by the end of the experimental period. Propionate was evident, but never exceeded 3 mM. The pH was stable throughout at 7.2 ± 0.1. The fermentation of 2% solids added cellulose showed acetate and pH profiles similar to the control (above) except acetate reached a maximum concentration of 18 mM and declined to trace amounts by day 18. Another difference was that propionate accumulated to a maximum of 8 mM on day 9, then declined to undetectable amounts by day 18. The acetate profile in the 4% added cellulose fermentation was nearly identical to the 2% fermentation, however, the propionate accumulation was greater. Propionate reached a maximum concentration of 21 mM on day 10 and then decreased to undetectable amounts by day

18. The beginning pH was 7.3 and the final pH was 7.1. The pH and volatile acid profiles of the 8dRT fermentation of 6% added cellulose were more variable than the other fermentations in this group. The data are shown in Fig. 3. It appeared that the pH was more sensitive to acetate concentration than to propionate or total volatile acid concentration.

The volatile acid and pH profiles for the fermenters on a 4d RT regime are shown in Fig. 4. The fermentations had not reached a true steady state even after 9 RT for cellulose additions and 12 RT for the control fermenter. The correlation between pH and acetate concentration was again evident.

Iron additions

Iron was added to feeds containing 0, 2, 4 and 6% added solids as cellulose to assess the possibility that limiting iron concentration may have been responsible for the oscillatory methane yields at high cellulose concentrations. It has been noted that iron additions to digesters at concentrations of 5 mM stimulated methane production (4), thus, ferrous chloride was added to digester feeds to provide 5 mM Fe^{++} in the digesters. Methane production from these fermentations was similar to fermentation without added iron and there was no significant difference in conversion efficiencies.

Discussion

This study indicated that the optimum mixture of cellulose and manure based on methane yield and solids conversion was obtained from feed that contained 41 to 54% of V.S. as added cellulose (Table 2). Fermenters which received feedstock of that composition produced

approximately 210 ml $CH_4 \cdot g^{-1}$ V.S., 300 ml $CH_4 \cdot g^{-1}$ cellulose and conversions of V.S. of approximately 50%. However, based on calculations of the percent of theoretical maximum yield attained, we tend to revise our estimate of the optimum mixture down to approximately 40% of V.S. as added cellulose. A theoretical maximum yield of methane can be calculated on the assumptions of 100% conversion of the added cellulose to methane, (414 ml $CH_4 \cdot g^{-1}$) and that the methane produced from the V.S. of manure remained constant with increased added cellulose. With these assumptions, 1, 2 and 3% solids cellulose fermentations produced 87, 81 and 82% of maximum yield, respectively, whereas, 4, 5 and 6% added solids cellulose produced 77, 74 and 68% of maximum yield, respectively. We assumed the optimum mixture remained the same regardless of the retention time based on the results of the 8d and 4d RT experiments.

It was interesting to compare the results of Hills (2) and Hills and Roberts (3) with our results. Their investigations of the effect of the C:N ratio on anaerobic digestion of manure plus glucose, cellulose or field crop residues indicate the optimum C:N ratio to be in the range of 25 - 32:1. Our range of C:N ratios was 20 - 45:1, with optimum found at 32:1 (3% added cellulose). We consider this to be good agreement considering the differences in digester conditions and operational factors.

We were concerned with the large variations in daily methane yields from these experiments. Calculation of the mean daily methane yield from several experiments did not "smooth" the data. Calculation of the mean squared error from the linear regression treatment of yields or from the sum of the squares of the residuals resulted in a value of 202 for the 6% added cellulose fermentations. However, for these same data

the mean squared error as determined by fitting a second order auto-regressive time series model to the daily methane yields was 76. This suggested that digesters may not have reached a true steady state or that population variations may have occurred. Further, it suggested calculating mean methane yields from a few determinations such as 5 consecutive days following 3 RT or a simple linear regression can be misleading when digesters are operated on high energy loads. The observations on the 4d RT fermentations also indicated that digesters receiving high loading concentrations did not reach steady state even after 9 RT as evidenced by volatile acid concentrations. Addition of iron did not remove the oscillatory behavior nor improve yields.

In summary, this study has shown that a manure-cellulose mixture with ca. 40% of V.S. as added cellulose provided the optimum mixture for methane production and solids conversion from digesters operated on semicontinuous flow. Secondly, the observations on fermentations at high loading rates indicated that digesters may not be capable of attaining a true steady state, i.e. both chemical and biological, under this mode of operation. Finally, the addition of iron provided no significant difference in digester performance, thus, the manure probably contained sufficient iron to provide optimum acetate conversion to methane.

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Acknowledgment

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We are grateful to Dr. John Amend for the use of computer facilities and consultation, and Drs. Martin Hamilton, Oscar Burt and Mr. Grier Jefferis for statistical analyses.

Table 1. Composition of dry matter of dairy cattle waste.

	% of dry matter
Volatile solids	83
Ether extract	2.6
Cellulose	31
Hemicellulose	12
Lignin	12.2
Total nitrogen (crude protein)	2.0 (12.5)[a]
Ammonia	0.5
Volatile acids	
Acetic	0.1
Propionic	Tr
Other	-[b]

[a] Data in parentheses indicate total nitrogen x 6.25 = crude protein.

[b] Not detected.

Table 2. Comparison of Average Daily CH_4 Production and Percent Conversion.

% Cellulose(g)	g VS	ml CH_4	ml CH_4/ g cellulose	ml CH_4/ g VS	% conversion[a]
		—— 16 d RT[b] ——			
0 -	0.17	25 ± 2	-	150 ± 12	30
1 (0.04)	0.21	36 ± 4	280 ± 50	170 ± 19	36
2 (0.08)	0.25	47 ± 5	280 ± 38	190 ± 20	42
3 (0.12)	0.29	61 ± 7	300 ± 42	210 ± 24	49
4 (0.16)	0.33	70 ± 10	280 ± 50	210 ± 30	50
5 (0.20)	0.37	80 ± 16	280 ± 70	220 ± 43	54
6 (0.24)	0.41	84 ± 14	250 ± 50	200 ± 34	51
		—— 8 d RT[c] ——			
0 -	0.33	44 ± 2	-	130 ± 6	25
2 (0.16)	0.49	69 ± 3	160 ± 6	140 ± 6	33
4 (0.32)	0.65	98 ± 9	170 ± 22	150 ± 14	38
6 (0.48)	0.81	92 ± 13	100 ± 23	110 ± 16	29
		—— 4 d RT[d] ——			
0 -	0.66	47 ± 3	-	71 ± 5	17
2 (0.32)	0.98	59 ± 16	38 ± 16	60 ± 8	15
4 (0.64)	1.30	102 ± 18	86 ± 23	78 ± 14	21
6 (0.96)	1.62	91 ± 17	46 ± 15	56 ± 10	16

[a] Percent conversion = $12(\text{mol gas}) \cdot 0.4^{-1}(\text{g VS})^{-1} \cdot 100$

[b] Daily averages were computed using data from days 17 through 56.

[c] Daily averages were computed using data from days 17 through 30.

[d] Daily averages were computed using data from days 16 through 36.

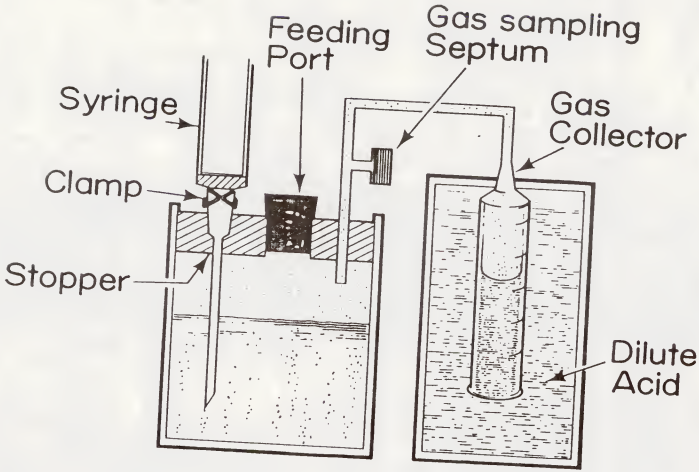
FIGURE LEGENDS

Fig. 1. Fermenter Diagram

Fig. 2. Daily Methane Yields. A. 16 d RT fermentation of 0, 2, 4, and 6% added cellulose; B. 8 d RT and C. 4 d RT for the same set of cellulose concentrations.

Fig. 3. The Profiles of pH, Propionate and Acetate from fermentation of 6% added cellulose on 8 d RT.

Fig. 4. The Profiles of pH, Propionate and Acetate from fermentation of 0, 2, 4 and 6% added cellulose on 4 d RT.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Septum
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Clamp
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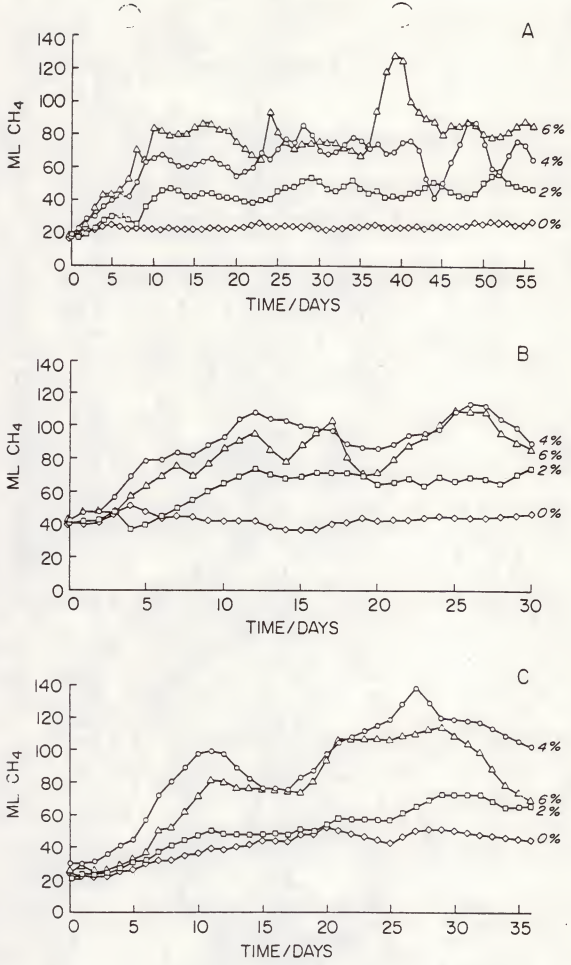
Dilute
Acid


A
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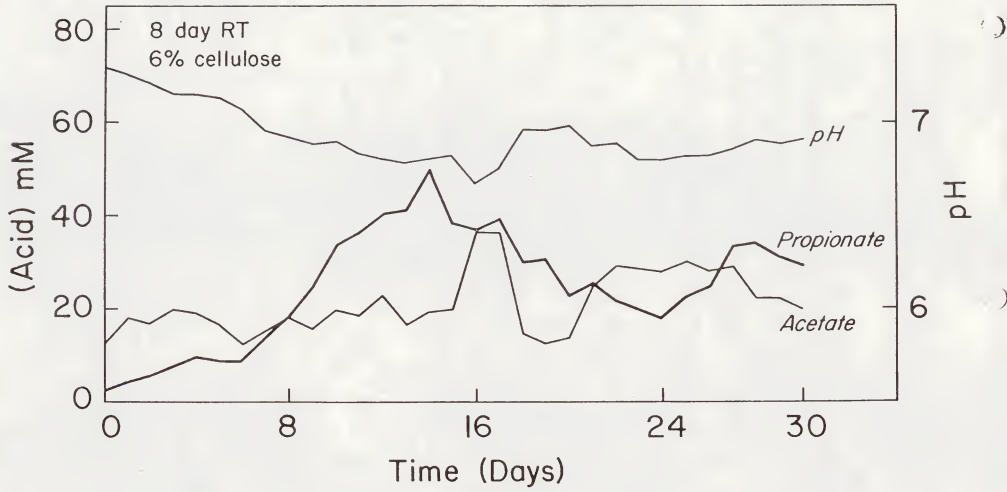
8 day RT
6% cellulose
80
60
40
20
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(Acid) mM
7
6
pH
pH
Propionate
Acetate
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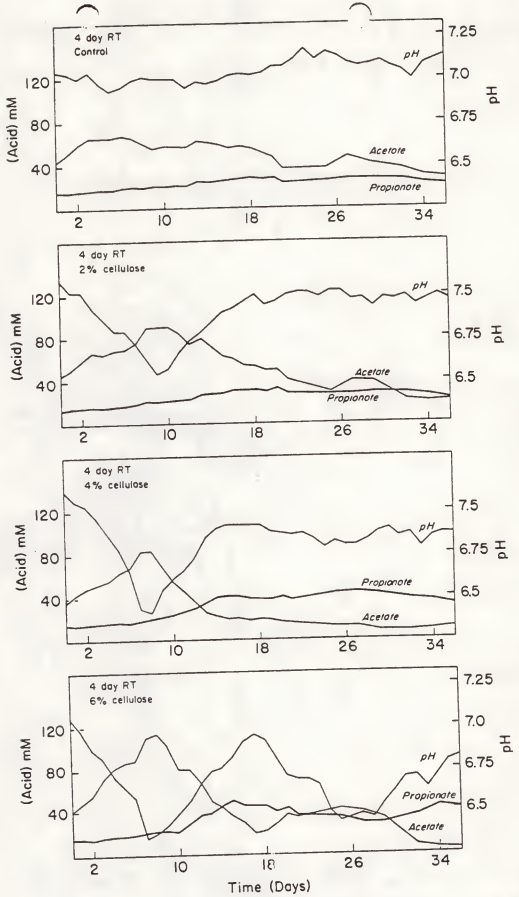
8
16
24
30
Time (Days)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Control
pH
Acetate
Propionate
(Acid) mM
pH
4 day RT
2% cellulose
4 day RT
4% cellulose
4 day RT
6% cellulose
Time (Days)

HYDROGEN ACCUMULATION IN ANAEROBIC DIGESTERS

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ABSTRACT

Anaerobic digesters fed daily with 5% total solids cattle manure containing 0.333 M glucose or 0.333 M glucose plus 0.169 M acetate (magnesium salt) were monitored for dissolved hydrogen concentration and volatile acid content as a function of time after feeding. The digesters accumulated hydrogen and volatile acids. The accumulation of propionate was concomitant with the accumulation of hydrogen in some of the experimental trials. These results indicated hydrogen inhibition of hydrogen formation may not be the primary cause of propionate accumulation in digesters receiving high energy loads in the form of glucose.

INTRODUCTION

In a recent study we examined the effects of adding large energy loads in the form of glucose and glucose plus acetate on anaerobic digestion of manure (4). The results of that study indicated that high concentrations of glucose caused digesters to accumulate large quantities of propionate and fail. The addition of acetate with high concentrations of glucose provided stable digestion. The present study was mainly concerned with evaluating whether hydrogen accumulation occurred with feeding high concentrations of glucose and if hydrogen inhibition of hydrogen formation might be the cause of propionate accumulation. Secondly, we wanted to compare hydrogen and volatile acid concentrations in fermentations of high concentrations of glucose versus high concentrations of glucose plus acetate. Thus, two series of experiments were run.

In the previous study fermenters which received 0.333 M glucose failed within 8 to 12 days after the introduction of 0.333 M glucose (4). This study focused on the days prior to failure, specifically days 1, 3, 5 and 7. In the former study samples were taken 24 hr after feeding but in this study we were concerned with the perturbations of hydrogen and volatile acid concentrations which might occur in the first 6 to 8 hrs after feeding. Five or 7 duplicate samples were taken throughout this time period. The first day the digesters received 0.333 M glucose or 0.333 M glucose plus 0.169 M acetate very little accumulation of either hydrogen or volatile acids occurred in the first 8 hrs. On days 3, 5 and 7 the digesters accumulated hydrogen and volatile acids within the first 2.5 hrs after feeding. Propionate and hydrogen increased in concentration simultaneously in some experiments, whereas, in

others hydrogen concentration had peaked and was declining while propionate continued to increase. These observations indicated that hydrogen inhibition could not have been the principal cause of propionate accumulation in these fermentations.

MATERIAL AND METHODS

The manure used daily in these experiments was collected from a holding area within two hours of deposition. The cattle were fed 40% grain mix and 60% hay. The grain mix contained 50% barley, 25% wheat, 10% soybean meal, 10% sugar beet pulp and 5% mustard meal. The manure composition is given in Table I. Total solids (T.S.) and volatile solids (V.S.) were determined by standard methods (1). The fiber content was determined as described by Goering and van Soest (2) and the nitrogen content by the Kjehldahl method. The manure was diluted with tap water to bring it to the desired total solids content. This mixture was subjected to 30 sec blending in a 3.8 l Waring blendor, then packaged in 1000 ml plastic bags and frozen. This feedstock was thawed and stored at 4° as needed. Glucose was obtained from Eastman and magnesium acetate was obtained from Baker. Both chemicals were reagent grade.

The digest used in these experiments was obtained from a three year old 3.2 l digester that had been maintained on a 16 day retention time (RT) at 37° on manure containing 5% total solids. Two anaerobic digesters were used in this experiment: (1) A 2 l Erlenmeyer flask which served as the primary digester for the experiment, and (2) An experimental fermenter (Fig. 1) which periodically received portions of digest from the Erlenmeyer fermenter.

The Erlenmeyer fermenter was maintained on a 16 day RT in a 37° water bath. The feed stock for the experimental period was 5% total manure solids with the following additions: (1) Sufficient glucose to give a 0.333 M glucose concentration in the manure, or (2) Sufficient magnesium acetate and glucose to give 0.169 M acetate and 0.333 M

glucose concentrations in the manure. Following feeding the digester was swirled for 15 sec to mix the contents. The fermenter was fed once per day. The experimental digester was given 320 ml portions (400 ml on day 0) from the Erlenmeyer flask one day before experimental samples were drawn. The digester was assembled, swept with CO_2 then digest added with a 50 ml syringe. It was maintained in a 37° water bath. On the day the experimental samples were to be drawn, digest was removed and an equivalent volume of 5% manure solids at 0.333 M glucose or 0.333 M glucose plus 0.169 M acetate was added by syringe. The digester was then swirled for 15 sec to mix contents. Two samples of known volume (20 ± 3 ml) were removed at each time point with 50 ml plastic syringes (B-D) from the exit port at the bottom of the experimental fermenter. These samples were analyzed by the following procedures.

The hydrogen concentration was determined by the procedures of Robinson et al. (5) with the following modifications: (1) 50 ml plastic syringes were substituted for the 50 ml glass syringe, (2) The samples were boiled for 15 minutes in the syringe, and (3) Shaking time was doubled, but vigorous shaking was avoided to minimize excessive foaming. The hydrogen concentration in the digest was estimated by the following equation:

$$[H_2] = 1000\ C_i\ V_G\ V_D^{-1}$$ where;

$[H_2]$ = moles/l digest

$C_i = [H_2]$ in GC injection volume (moles/ml)

V_G = gas volume above aqueous NaOH, NaCl column (ml)

V_D = digest sample volume (ml).

The equation for determining hydrogen concentration described by Robinson et al. (5) was not used because an accurate graphical determination of the Bunsen coefficient for digest could not be obtained. Our estimation will therefore be low by some value between 0 and 5%. (For example, our estimate would be 1.3% low using the Bunsen coefficient for water and a 20 ml digest sample in a 50 ml syringe working volume.) GC analysis for hydrogen content was performed on duplicate samples. The hydrogen concentration reported is the average of a minimum of four (2 each sample) analyses for each set of duplicates.

Analysis of Volatile Acids

One and one half ml portions of digest samples used in the above procedure were centrifuged. Five hundred µl portions of the obtained supernatants were acidified with 100 µl 25% metaphosphoric acid. The acidified samples were incubated approximately 30 minutes and then analyzed by gas chromatography using a 1.8 m x 2 mm glass column packed with 10% SP-1200, 1% phosphoric acid (Supelco). Column operation was isothermal (115^{o}) with a flow rate of 30 ml/min nitrogen. Detection was accomplished by flame ionization and a Hewlett Packard 3380A integrating recorder.

RESULTS

These experiments were designed to determine the concentration of dissolved hydrogen and volatile acids in digest during the hours immediately after feeding large daily quantities of glucose or glucose plus acetate. The concentrations of hydrogen, propionate and acetate in digesters receiving 0.333 M glucose or 0.333 M glucose plus 0.169 M acetate (magnesium salt) are shown in Figs. 2 and 3 respectively. The data points shown were averages of duplicates. Error bars are shown where variation between duplicates occurred, otherwise the variation was too small to depict. The filled symbols in the propionate data are calculated concentrations based on dilution by the feed. Both fermentations produced slight accumulation of hydrogen and propionate on the first day of the regime in the first 8 hr period. Propionate increased from trace amounts to 3 to 5 mM in both fermenters. Hydrogen concentrations were somewhat greater in the fermentation of glucose as the sole addition to the manure feed. On days 3, 5 and 7 there was a more immediate and more pronounced response after feeding. The accumulation of propionate and hydrogen was concomitant on days 1, 5 and 7 in both fermentations. Propionate continued to accumulate after hydrogen had attained a maximal concentration and while hydrogen concentration was declining on day 3 of fermentation of 0.333 M glucose. The ability of fermenters to accumulate hydrogen within 30 min after feeding increased on each subsequent sampling day, with the exception of day 7 in the digester receiving acetate.

DISCUSSION

The observation that the fermenters apparently produced more hydrogen with each subsequent feeding and the lack of the production of large amounts of hydrogen within the first 8 hrs on the first day suggested that enrichment of hydrogen producing fermentative bacteria occurred in these fermentations. The digesters definitely showed increased capacity to accumulate hydrogen. The lack of the large increase in hydrogen concentration on day 7 in the fermentation receiving added acetate suggested that hydrogen utilization activity may have increased in comparison with the fermentation without added acetate.

The simultaneous increase in propionate and hydrogen in both fermentations indicated that hydrogen inhibition of its own formation could not have been the principal cause of the propionate accumulation. If hydrogen inhibition were the principal cause of propionate accumulation then propionate accumulation should have commenced after peak hydrogen accumulation. These results indicate that hydrogen formation rates were increasing while propionate accumulation occurred. This suggests that hydrogen formation rates were not fast enought to supply NAD^+. The hydrogen forming fermentative bacteria thus sought an alternative electron sink, viz. propionate. This is speculation at this point, however, it was interesting to approximate the $NADH:NAD^+$ ratio in fermenters that had not been stressed by a high energy load in the form of glucose, i.e. at steady state on manure feed. The hydrogen concentration in these fermenters was 10^{-7}M and the hydrogen ion concentration was 4×10^{-8} (pH = 7.4). If one assumes a ΔG value of -0.2 kcal/mol., then the $NADH:NAD^+$ ratio would be approximately 5000:1. This suggests the NAD^+ supply in hydrogen producing bacteria

may be limiting. It is also possible that enrichment of bacteria which have a tendency to produce propionate from glucose could have occurred. This could also explain the simultaneous increase in hydrogen and propionate. We would also like to point out that other acids that could serve as electron sinks may be observed to accumulate, viz. lactate or succinate, depending on the predominating bacterial species.

The observations obtained in this study are consistent with those of Kaspar and Wuhrmann (3). They estimated the maximal rate of hydrogen utilization to be greater than that of either propionate oxidation or acetate utilization in digesting sludge. Thus it seems unlikely that hydrogen utilization would be rate limiting in the overall process of converting waste material to methane. These observations are important in considering means to increase the efficiency of methanation. One would approach the problem of increasing efficiency of methanation differently if one assumed the methanogen population presented limitations rather than the fermentative or the acetogenic populations.

ACKNOWLEDGEMENT

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TABLE I. Composition of Dry Matter of Dairy Cattle Waste and Volatile Acid Concentration.

	% of dry matter
V.S.	83
Ether Extract	2.5-2.8
Cellulose	31
Hemicellulose	12
Lignin	12.2
Total Nitrogen (crude protein)	2.0 (12.5)[a]
Ammonia	0.5
Volatile acids; acetic	27.4 ± 10.4 mM
propionic	2.4 ± 0.4 mM
butyric	0.5 ± 0.1 mM
valeric	0.1 ± 0.1 mM

LEGENDS FOR FIGURES

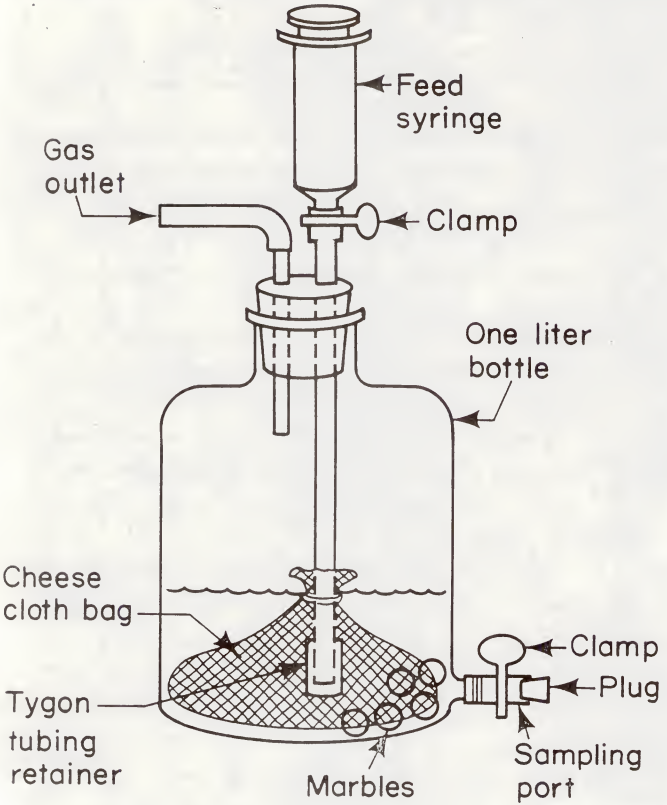
Fig. 1. Experimental fermenter. The special features of this apparatus are the cheesecloth bag attached to the feed input tube, which confines the particulates; the tygon tubing that prevents the cheesecloth from slipping off the feed tube; the marbles, which prevent clogging of the sampling port, and the sampling port itself.

Fig. 2. The accumulation of hydrogen, propionate and acetate following feeding of 0.333 M glucose. Propionate concentration (Δ), acetate concentration (□) and hydrogen (o).

Fig. 3. The accumulation of hydrogen, propionate and acetate following feeding of 0.333 M glucose and 0.169 M acetate (magnesium salt). Symbols are the same as Fig. 2.

Footnote [a] for Table 1.

[a] Data in parenthesis indicate total nitrogen x 6.25 = crude protein.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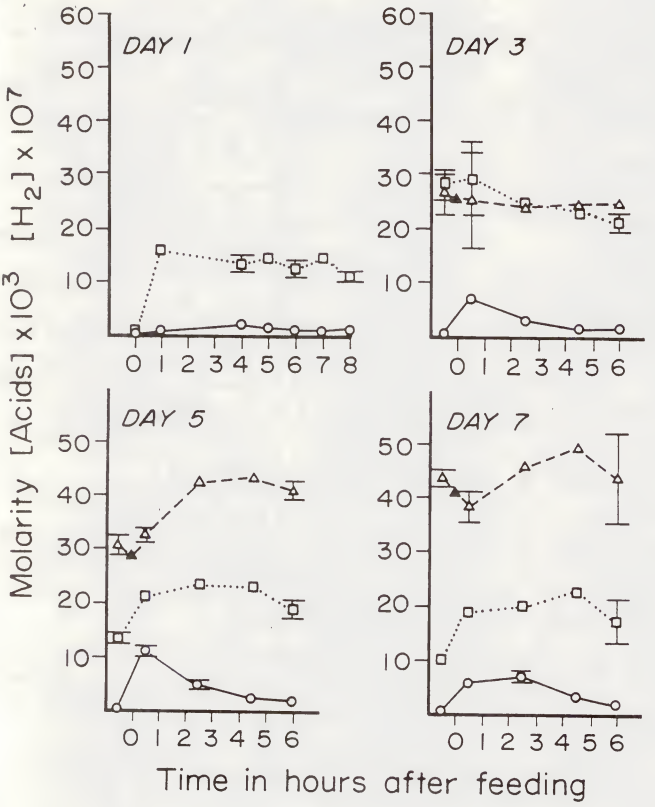
Gas outlet
Clamp
One liter bottle
Cheese cloth bag
Clamp
Plug
Tygon tubing retainer
Marbles
Sampling port

DAY 1
DAY 3
DAY 5
DAY 7
Molarity [Acids] x 10^3 [H2] x 10^7
Time in hours after feeding


DAY 1
DAY 3
DAY 5
DAY 7
Molarity [Acids] x 10^3 [H2] x 10^7
Time in hours after feeding

INTRODUCTION

Methane production from anaerobic digestion of wastes is being considered as a source of renewable energy. Digester operation at higher than normal loading rates has been investigated in an attempt to increase the rate of methane yield (21) and the economic feasibility. When digesters are operated at too high a loading rate they often exhibit instability and less than optimum methane yields. Volatile acids accumulate and pH decreases under these conditions. The stability of anaerobic digestion hinges on the ability of the microbial ecosystem to produce and convert volatile acids to gases. Previous investigations (1, 21) have shown that propionate accumulates in stressed fermenters. According to Kroeker et al. (12) this represents metabolic imbalance. The cause of propionate accumulation in the above studies has not been experimentally identified. This investigation was concerned with identifying factors which lead to metabolic imbalance under high energy loads.

Methanogenesis results from complex metabolic interactions of three classes of anaerobic bacteria (2). Fermentative bacteria hydrolyze polymeric substances to simple molecules, e.g. cellulose is converted to sugars. These are catabolized to volatile acids, CO_2 and H_2. Acetogenic bacteria convert volatile acids such as propionate , butyrate and others to acetate, CO_2 and H_2. Methanogenic bacteria produce methane primarily from acetate, CO_2 and H_2 (9). Methanogenic bacteria have profound effects on the metabolism of the other bacteria (3, 4, 8, 13, 16, 19, 22, 23). From these studies it is apparent that the mechanism used for electron disposal by fermentative bacteria influences their volatile acid production.

Accumulation of volatile acids such as propionate presents the anaerobic ecosystem with a difficult task with regard to methane production. The oxidation of propionate to acetate, $\Delta G^{o'} = 18.2$ kcal/mol., is more unfavorable than the production of H_2; $\Delta G^{o'} = 4.3$ kcal/mol. (20). The work of Andrews and Pearson (1) brought attention to the accumulation of propionate in stressed fermenters. The dissipation of accumulated propionate was slow. Acetate accumulated also but it disappeared quickly. Varel et al. (21) observed that the accumulation of propionate in stressed fermenters preceded the accumulation of other volatile acids.

In a study by Kaspar and Wuhrmann (10) the kinetics of "acetate splitting", propionate oxidation and H_2 utilization were measured. They concluded "acetate splitting" was the slowest and H_2 utilization was the fastest process in digesting sludge at steady state. However, they estimated the maximal velocities for acetate, propionate and H_2 utilization as 0.63 mmol/l·hr, 0.16-0.3 mmol/l·hr, and 103 mmol/l·hr respectively. In another study by Kaspar and Wuhrmann (11) they suggested the overall limiting factor in methane production was the oxidation of propionate.

Preliminary experiments conducted in our laboratory indicated propionate accumulated when high concentrations of glucose were fermented with manure. No acetate accumulation was evident in these experiments. These observations in conjunction with the observations that acetate accumulates later than propionate and dissipates more readily lead us to believe that methanogenic activity of acetate splitting was not a rate limiting factor. Since propionate accumulation indicates an imbalance in the metabolic processes, we were concerned with

possible causes.

One series of experiments was designed to establish the glucose concentration (energy level) which caused an imbalance. Anaerobic digesters were fed cattle manure to which several concentrations of glucose were added. Digesters which received an energy load which caused metabolic imbalance would most probably exhibit the metabolic activities that were limiting. In a second series of experiments acetate was added along with glucose. Two levels of glucose concentration were chosen; one high enough to cause destabilization and one below that level. The rationale for this second series was that added acetate would exacerbate the destabilization caused by the high energy load if the methanogenic activity of acetate utilization was rate limiting. Secondly, the fermentations at the lower glucose concentration may destabilize where they had been stable with glucose alone.

A third series of experiments was designed to assess whether the effects of magnesium acetate observed in the second series of experiments were due to acetate or magnesium.

The performance of the small digesters was found to be equivalent with the large (3.2 l) digester. The advantages of the small digesters were less materials were required for the studies and more digesters could be accomodated in each experiment. Evidence that manipulations with small digesters did not introduce variations in results was shown by the stable performance of the control fermenters. Further, the 4 ml of digest removed daily provided adequate sample to perform all analyses. A control fermenter of 5% total solids manure was operated with each set of experiments.

These experiments indicated high concentrations of glucose

produced unstable fermentation and digester failure, but added acetate, tended to stabilize the digestion. These findings led us to conclude that acetate splitting was not rate limiting. Thus, under high energy loads it is probable that the metabolism of propionate and higher molecular weight volatile acids by the acetogenic bacteria becomes critical to methanation.

MATERIALS AND METHODS

Manure and Digest

The manure was obtained from the Montana State University dairy. The manure collection and analysis have been previously described (17). The composition of the manure used in the experiment is shown in Table 1. The digest used in starting the experimental fermenters was obtained from a larger digester (working volume of 3.2 l) which had been maintained at 37° on a 16 day retention time (RT) for over three years on manure containing 5% total solids.

Fermenters

The fermenters were made from 100 ml wide mouth lyophilization flasks (Thermovac) fitted with a #9 three hole rubber stopper (Fig. 1). One hole was fitted with a y-tube for gas sampling and gas collection. The gas was collected in a calibrated gas receiving vessel. The second hole was fitted with a 10 ml syringe to allow additions of feed and removal of digest. The third hole was normally plugged and opened only during feeding and removal of digest to allow pressure equilibration. The working volume was 64 ml. A 16 day RT was chosen based on other investigations (1, 6).

Conditioning and Daily Procedure

The fermenters were maintained in a water bath at 37° on a 16 day RT with 5% total solids manure as feed until pH, gas volume and gas composition were similar in all fermenters. The fermenters were then fed manure containing 0.056 M, 0.111 M, 0.167 M, 0.222 M, 0.278 M or 0.333 M glucose in the feed. The glucose concentration in the digester is then 1/16 of that value. In the remainder of the text we refer to added substrates in terms of concentration in the feed.

In a second series of experiments the fermenters received manure plus 0.111 or 0.333 M glucose and 0.169 M acetate (magnesium salt). In the third series of experiments fermenters received manure containing 0.333 M glucose and one of the following; no additions, 0.169 M acetate (potassium salt), 0.169 M acetate (magnesium salt) and 0.084 M $MgCl_6 \cdot 6H_2O$. Each day the gas volume and composition were determined as described below. The fermenters were swirled to suspend particulates, 4.0 ml of digest was removed and 4.0 ml of the appropriate feed was added. After resealing, the fermenters were again swirled to mix the feed and digesta. The pH and volatile acid composition were determined on each digest sample. The acids of major interest were acetate, propionate, butyrate, lactate, isobutyrate, isovalerate and valerate. Another compound of interest was ethanol. Lactate was observed in only a few instances in minor amounts and in those fermenters lactate disappeared usually by the subsequent day. In experiments performed in this laboratory (the data are not presented here) lactate was produced from glucose but usually reached a maximum concentration 3 hrs. after feeding and was virtually absent after 24 hrs. Ethanol was observed in one experiment and was also noted to be transient. In all experiments performed no attempt was made to control the pH.

Analyses

Gas volume was determined by water displacement using acidified water. Gas composition and volatile acid profiles were determined by the gas chromatographic procedures described by Robbins et al. (17). Gas volume and composition were determined every 12 hrs or daily as needed. Each time the analyses were performed the fermenters were thoroughly mixed by swirling. Gas composition for the glucose additions

was determined only once but gas production was recorded at 12 hr intervals and released. Thus, CH_4 production was reported in these experiments for the latter 12 hr period of the day. Ammonium ion concentrations were determined enzymatically by the method of Robbins and Weber (18). Bicarbonate concentrations were calculated using equation 1.

$$[HCO_3^-] = K_a \cdot K_h \cdot P_{CO_2} \cdot [H^+]^{-1} \qquad \text{eq. (1)}$$

Where; $K_a = 10^{-6.1}$; $K_h = 10^{-1.42}$ and P_{CO_2} was in atmospheres.

Chemicals

All chemicals were reagent grade. Magnesium acetate and potassium acetate were obtained from J. T. Baker and glucose was obtained from Eastman.

RESULTS

In this study we examined the effects of several energy loads of glucose or glucose plus acetate on methane yields and volatile acid profiles of anaerobic digesters. The glucose concentrations chosen provided increments from modest to very high energy loads. High energy additions caused significant changes in the volatile acid profiles and methane yields. We viewed these perturbations from steady state as being appropriate for examining the rate limiting processes.

I. The effects of added glucose on anaerobic digestion

A. Methane production

Seven fermenters were established as described above to assess the amount of glucose which caused failure. The fermenters received 5% by weight manure solids and glucose at concentrations of 0, 0.056, 0.111, 0.167, 0.222, 0.278 and 0.333 M in the feed. Methane production from the fermenters is shown in Fig. 2. Methane production from the fermenter receiving no glucose was 23 ± 1 ml/day (mean ± 1 standard deviation). Methanation ceased (fermenter failure) and pH decreased in fermenters that received 0.222, 0.278 and 0.333 M glucose in the feed. Methane yield fell below 4 ml/day on days 16, 11 and 8 respectively. The fermenters that received 0.056, 0.111 and 0.167 M glucose remained productive and pH was stable.

B. Volatile acid and pH profiles

The fermenters receiving glucose additions showed minor temporary increases in acetate concentration during the first three days of glucose feeding, with a maximum concentration of < 15 mM attained in the fermenter receiving 0.333 M glucose. After three days, the fermenters receiving 0.056, 0.111, 0.167, 0.222 and 0.278 M glucose additions

showed decreases in acetate concentration to pre-glucose feeding levels. The digesters receiving 0.222, 0.278 and 0.333 M glucose all showed increases in acetate concentrations which preceded cessation of methanation by one to three days. The acetate concentration in these three fermenters was highest on the day the fermenters were terminated. The concentrations of acetate were 20, 30 and 35 mM respectively. The propionate profiles are depicted in Fig. 3. The rates of accumulation are proportional to the glucose concentration. Propionate accumulated in all fermenters receiving glucose but decreased to nondetectable levels in fermenters that received 0.056 and 0.111 M glucose. Propionate reached the highest concentration in the fermenter that received 0.222 M glucose and propionate concentration began to decrease in the fermenter that received 0.278 M glucose just prior to failure at a rate exceeding the wash-out. The fermentation of 0.167 M glucose accumulated propionate through the first 16 days and appeared to be capable of acclimating since the methane production was high (57 ± 5 ml/day) and the pH was stable. This fermention was carried out for 65 days. The volatile acid content decreased to trace amounts by day 18, the pH remained stable and the methane production remained near 57 ml/day. The pH for fermenters that received 0.222, 0.278 and 0.333 M glucose began at 7.4 and slowly declined to 7 ± 0.2 one to three days prior to failure. At these times acetate concentrations increased and the pH declined rapidly from 7 ± 0.2 to 5.5 ± 0.3. The pH in the fermenters receiving 0.056, 0.111 and 0.167 M glucose remained within the range 7.2 to 7.5.

C. Ammonium ion concentrations

Ammonium ion concentrations were evaluated in fermenters receiving

0.222 M and 0.333 M glucose in addition to manure. The concentration of ammonium ion decreased in both these fermenters from 25 ± 0.5 mM to 19 ± 0.5 mM for these experiments.

D. Bicarbonate concentrations

The bicarbonate concentrations were calculated as described in methods. Comparisons of bicarbonate:volatile acid ratios were made for the three fermenters that failed. In these experiments the bicarbonate:propionate ratios were, 35, 12, 3.5 for 0.222, 0.278 and 0.333 M glucose respectively at the time methane production began to decline. The pH values at these times were 7.56, 7.08 and 6.95 respectively.

II. The effects of glucose and acetate additions on anaerobic digestion

A. Methane production

Magnesium acetate was added to fermenters receiving 0.111 and 0.333 M glucose to determine whether addition of acetate would exacerbate the destabilization caused by glucose additions.

Glucose additions of 0.111 and 0.333 M in the feed were chosen as representative of stable and unstable fermentations as demonstrated in the previous experiments. Methane production from digesters receiving glucose and acetate additions is shown in Fig. 4. The greatest methane yield was obtained from 0.333 M glucose and 0.169 M acetate. The acetate addition improved the methane yield at both 0.333 and 0.111 M glucose. The methane production from 0.333 M glucose and 0.169 M acetate attained ca. 120 ml per day after 20 days (Fig. 4 includes only the first 16 days). The addition of acetate provided methane production throughout the experimental period in contrast to the fermentation of 0.333 M glucose alone. We want to emphasize the consistent performance of the control depicted in Fig. 4. This performance was

typical of all the controls run for each set of experiments and indicated the fermenter design and the daily manipulations provided a mechanically sound and reproducible procedure.

B. Volatile acid and pH profiles

The fermenter receiving 0.333 M glucose showed accumulation of propionate to a maximum value of 180 mM on day 9. Acetate remained near 10 mM for the first 8 days, then increased to 34 mM on day 10. Butyrate was first evident on day 8 and increased to 6 mM on day 10. No other acids were present in more than trace amounts. The fermenter receiving 0.333 M glucose plus 0.169 M acetate also accumulated propionate but the maximal value was 43 mM on day 13. Propionate then decreased to 33 mM by day 16. Acetate was highest at 26 mM on day 10 and subsequently decreased to 8 mM $\pm$ 3 mM. The concentrations of other acids did not exceed trace amounts. The volatile acid content of fermenters receiving 0.111 M glucose and 0.111 M glucose plus 0.169 M acetate were comparable to the volatile acid content of the control fermenter (i.e. 5% manure solids only). The pH profile in the fermenter receiving 0.333 M glucose was similar to the fermentation of 0.333 M glucose in the first series of experiments. The pH began at about 7.5, slowly decreased until acetate began to accumulate just prior to failure and then declined sharply as acetate concentration increased. The pH in the other fermenters in this series remained relatively stable throughout the experimental period.

III. The effects of magnesium and acetate additions on anaerobic digestion of glucose

A. Methane production

The addition of magnesium acetate to fermentations of manure and

high concentrations of glucose resulted in increased stability and methane production. The role of acetate and magnesium in the observed increased stability and methane production was tested by additions of magnesium acetate, potassium acetate or magnesium chloride to digesters receiving 0.333 M glucose. Fig. 5 shows methane production from these fermentations.

Methane production was similar for the first four to five days for the four fermenters receiving 0.333 M glucose. Methane production then decreased rapidly in the fermenters receiving 0.333 M glucose and 0.333 M glucose plus 0.084 M $MgCl_2 \cdot 6H_2O$. Methanation ceased on days 12 and 9 respectively. The fermenters receiving 0.169 M acetate as the Mg^{++} or K^+ salt showed increasing methane production and peaked at around 140 ml/day on days 18 and 20 respectively. The fermenter receiving potassium acetate remained productive throughout the 53 day experimental period, but the fermenter receiving magnesium acetate showed decreasing methane production with only 10 ml produced on day 53. A control fermenter receiving only 5% manure solids was stable throughout this period.

B. Volatile acid and pH profiles

The volatile acid content and pH of these fermentations are depicted in Fig. 6. Propionate accumulated in all fermenters receiving glucose. Propionate accumulation was evident through the experimental period in fermenters receiving 0.333 M glucose and 0.333 M glucose plus 0.084 M $MgCl_2 \cdot 6H_2O$. Propionate concentration increased for only 4 to 5 days in fermenters receiving acetate additions and then decreased to trace levels. In the fermentation of manure, glucose and magnesium acetate, propionate again began to accumulate after 31 days. Acetate began to

accumulate in this fermentation shortly after methane production (Fig. 5) began to decline. Significant amounts of butyrate accumulated also but not until day 33. In the fermentation with potassium acetate the propionate level remained low throughout the experimental period after the initial increase. Acetate remained relatively low throughout the experimental period with fluctuations between 2 and 11 mM. The pH of these fermentations was similar to that of the previous experiments. The fermenters which failed exhibited a decline in pH throughout with a very rapid decline that correlated with the accumulation of acetate. The fermenter that received magnesium acetate showed a late destabilization with acetate accumulation and pH change again correlated, however, acetate accumulation preceded propionate accumulation in this fermenter. The pH of the fermenters receiving potassium acetate and 5% manure varied between 7.3-7.5.

DISCUSSION

The first series of experiments evaluated the energy load required to cause serious imbalance among the metabolic interrelationships or the microbial populations. Imbalance was considered to exist if either methane production decreased or if accumulation of volatile acids occurred. Fermenters which received 0.167, 0.222, 0.278 and 0.333 M glucose in the feed exhibited imbalance as indicated by the data given in Figs. 2 and 3. Fermenters which received 0.056 and 0.111 M glucose were stable. The fermenter which received 0.167 M glucose was not stable in the first 16 days, the period used for comparative purposes. However, the fermenter was able to acclimate to this additional energy load. The fermenters that obtained 0.222, 0.278 and 0.333 M glucose all failed. The propionate profiles shown in Fig. 3 indicated these fermentations were severely destabilized by the accumulation of propionate. Acetate accumulated in these fermenters also, but only a day or two prior to cessation of methane production. The decline in methane production began 3 to 6 days prior to acetate accumulation. These observations in conjunction with the accumulation of propionate from the beginning of the experiment, suggest that a disproportionate amount of the glucose was converted to propionate rather than acetate. Nevertheless, acetate, unlike propionate, did not show accumulation. Thus, the digesters could utilize the acetate produced each day until they approached failure. These observations could be explained hypothetically in a number of ways. Propionate may be produced as an alternative means to regenerate NAD^+ in addition to the reaction shown in equation 2. This may be necessary if the reaction below is

$$NADH + H^+ \rightleftharpoons NAD^+ + H_2 \qquad \text{eq. (2)}$$

relatively slow compared to glucose metabolism to pyruvate or if H_2 concentration increases to inhibitory concentrations. In either event, glucose would be diverted from production of acetate. Secondly, in these experiments it is possible that enrichment of bacterial populations, which have the propensity to produce propionate from glucose, could have occurred. Thus, the availability of glucose for conversion to acetate would be reduced. Thirdly, acetate may have accumulated to concentrations which were inhibitory to propionate oxidation (11) within a few hours after feeding. This caused propionate to accumulate. In the remainder of the day the acetate was completely utilized whereas the propionate was not. In the first possibility either the production of NAD^+ by eq. 2 or the methanogenic activity of H_2 utilization would be rate limiting. In the second possibility, an imbalance of microbial populations might produce the excess propionate. The rate limiting process here would be the oxidation of propionate. In the third possibility, the methanogenic activity of acetate utilization may be initially rate limiting causing an accumulation of propionate. The final limiting process here would be the oxidation of the propionate.

The second series of experiments was designed to test whether acetate utilization was overburdened or under supplied. The effect of acetate addition on methane production is shown in Fig. 4. The fact that the fermentations of added acetate enhanced methane production supports the suggestion of inadequate supply of acetate. Secondly, the fermenter that received 0.333 M glucose plus 0.169 M acetate accumulated less propionate than the fermenter that obtained 0.333 M glucose, i.e. maximum concentrations of 43 vs 180 mM. This observation suggested that the additional acetate provided for greater

methanogenic activity which in turn reduced the amount of propionate accumulated. Perhaps increased acetate utilization was accompanied by increased H_2 utilization, thus propionate was oxidized more readily.

In most fermentations where acetate accumulated there was a correlation between acetate concentration and pH. The fermentation of 0.333 M glucose plus 0.169 M acetate, magnesium salt, was an exception. The pH in that experiment increased throughout the experiment while the acetate increased to a maximum of 26 mM and then decreased to ca. 8 mM. This caused concern over some unforseen effects due to magnesium.

A third series of experiments was performed to determine whether the above observations were principally due to acetate or magnesium. Methane production from this third series is shown in Fig. 5. Since fermentation of glucose and magnesium chloride failed earlier than glucose as the sole addition, and since, the fermentations of 0.333 M glucose and added acetate produced methane for an extended period of time the acetate must have provided added stability. The fermentation of glucose and magnesium acetate did destabilize for reasons not known. The mechanism of destabilization was clearly different than with glucose as evidenced by a large increase of acetate long before methanation ceased (Fig. 6). Methanation ceased on day 60 in this fermentation. The fact that both magnesium and potassium acetate fermentations exhibited an initial, but brief, accumulation of propionate, whereas the fermentation with magnesium chloride accumulated propionate until the digester failed, also suggested that acetate had a beneficial effect.

These studies have shown that large amounts of soluble sugar can

destabilize and cause fermenter failure. Secondly, acetate additions with large energy loads in the form of glucose exhibited a sparing effect against failure. The observations on the effects of glucose are important when considering pretreatments such as hydrolysis as a means of circumventing the rate limitations of cellulose hydrolysis (7). The observations of the effect of added acetate point out the importance of maintaining adequate methanogenic activity for the overall efficiency of anaerobic digestion. Thus, a closer look at two stage digestion versus single stage digestion is warranted (5, 7).

Kroeker et al. (12) and McCarty (14) discussed the toxicity of NH_4^+ and McCarty and McKinney (15) examined the toxicity of propionate. Kaspar and Wuhrmann (11) suggested the importance of the HCO_3^-:volatile acid ratio for stable digestion. Careful inspection of the concentrations of NH_4^+, H^+, HCO_3^-, and volatile acids did not provide any insight as to the critical balance among these species. Thus, the reasons for fermenter failure are unclear. Secondly, this study did not provide concrete evidence as to how acetate additions provided stability under destabilizing concentrations of glucose.

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LEGENDS FOR FIGURES

Fig. 1. Diagram of small fermenters.

Fig. 2. A comparison of methane production from the last 12 hrs. of a 24 hr. period is shown. Glucose concentrations were as indicated.

Fig. 3. Propionic acid concentrations in fermenters that received 0.056, 0.111, 0.167, 0.222, 0.278 and 0.333 M glucose.

Fig. 4. A comparison of daily methane production from glucose as the sole addition with manure feed versus glucose plus acetate addition.

Fig. 5. The effect of magnesium versus acetate on daily methane production. A. Methane production from 5% T.S. manure = control; 0.333 M glucose and 0.333 M glucose plus 0.169 M acetate (potassium salt). B. Methane production from 0.333 M glucose plus $MgCl_2 \cdot 6H_2O$ (0.084 M Mg) and 0.333 M glucose plus 0.169 M acetate (magnesium salt).

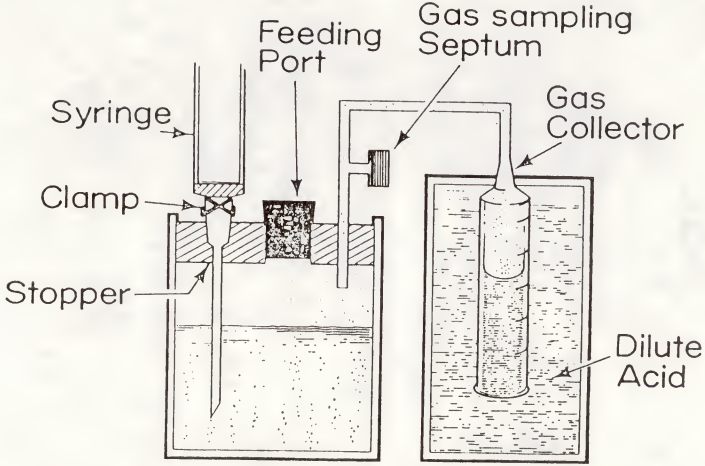
Fig. 6. Volatile acids and pH in fermenters that received; A. 0.333 M glucose; B. 0.333 M glucose plus 0.084 M Mg^{++}; C. 0.333 M glucose plus 0.169 M potassium acetate; and D. 0.333 M glucose plus 0.169 M acetate (magnesium salt). The pH is indicated by the dashed line and the acids are indicated in the graphs.

Table 1. Composition of dry matter of dairy cattle waste.

	% of dry matter
Volatile solids	83
Ether extract	2.6
Cellulose	31
Hemicellulose	12
Lignin	12.2
Total nitrogen (crude protein)	2.0 (12.5)[a]
Ammonia	0.5
Volatile acids	
Acetic	0.1
Propionic	Tr
Other	-[b]

[a] Data in parentheses indicate total nitrogen x 6.25 = crude protein.

[b] Not detected.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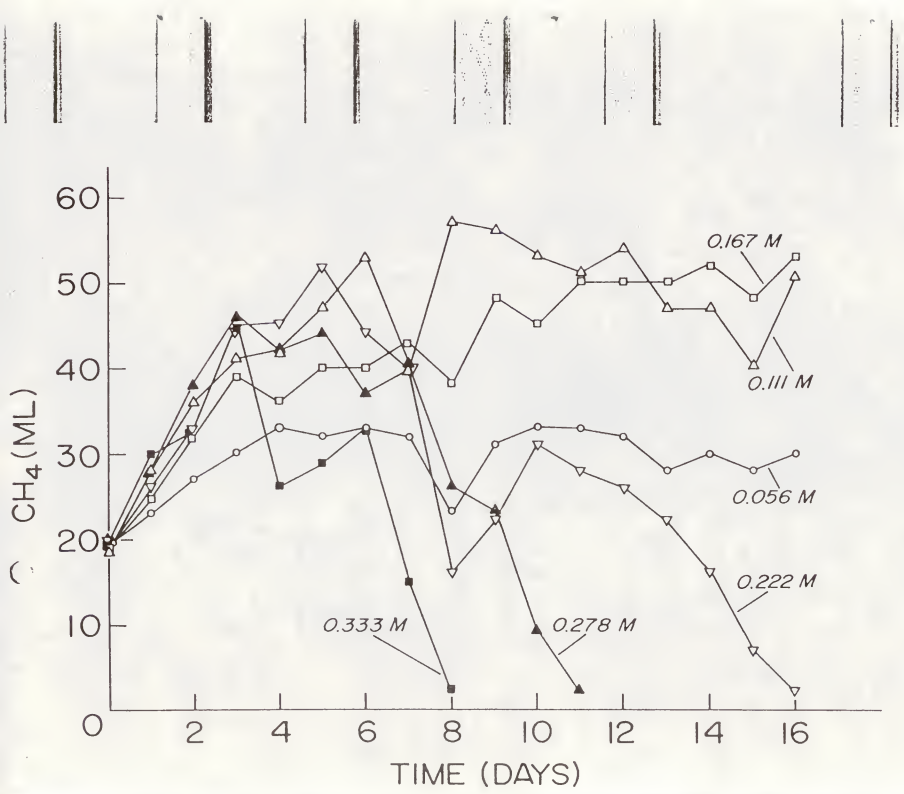
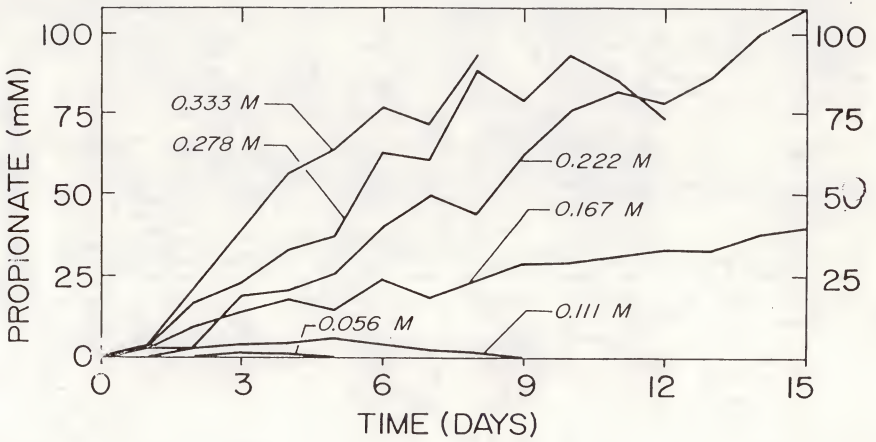
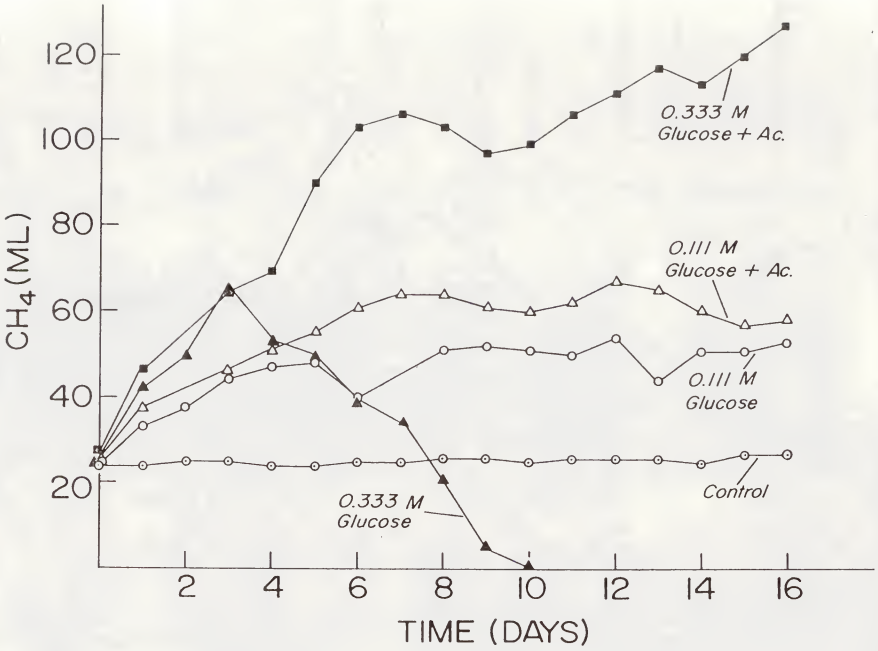
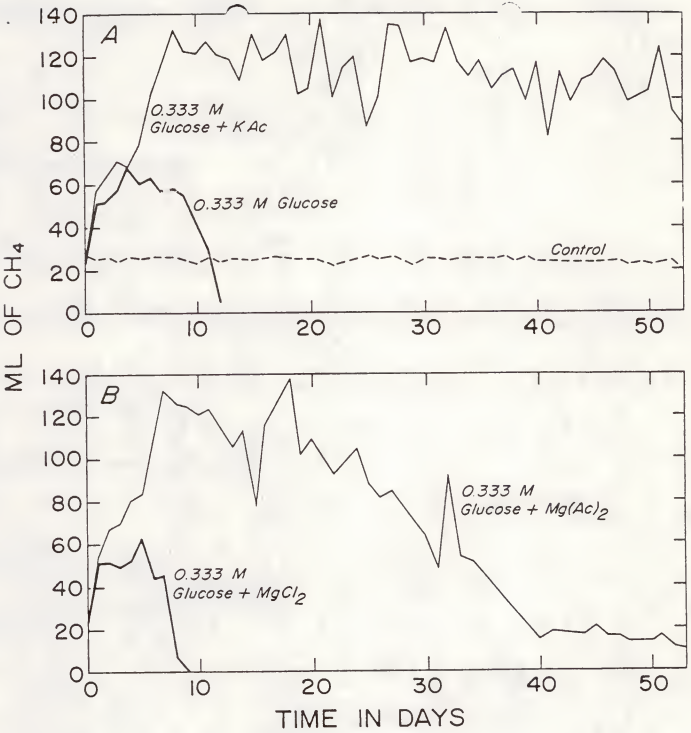

60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
CH4 (ML)
TIME (DAYS)
0.167 M
0.111 M
0.056 M
0.222 M
0.278 M
0.333 M


PROPIONATE (mM)
100
75
50
25
0
0.333 M
0.278 M
0.222 M
0.167 M
0.056 M
0.111 M
0
3
6
9
12
15
TIME (DAYS)


120
100
80
60
40
20
CH_4 (ML)
2
4
6
8
10
12
14
16
TIME (DAYS)
0.333 M
Glucose + Ac.
0.111 M
Glucose + Ac.
0.111 M
Glucose
Control
0.333 M
Glucose


A
0.333 M Glucose + KAc
0.333 M Glucose
Control
B
0.333 M Glucose + Mg(Ac)2
0.333 M Glucose + MgCl2
ML OF CH4
TIME IN DAYS
0
10
20
30
40
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0
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40
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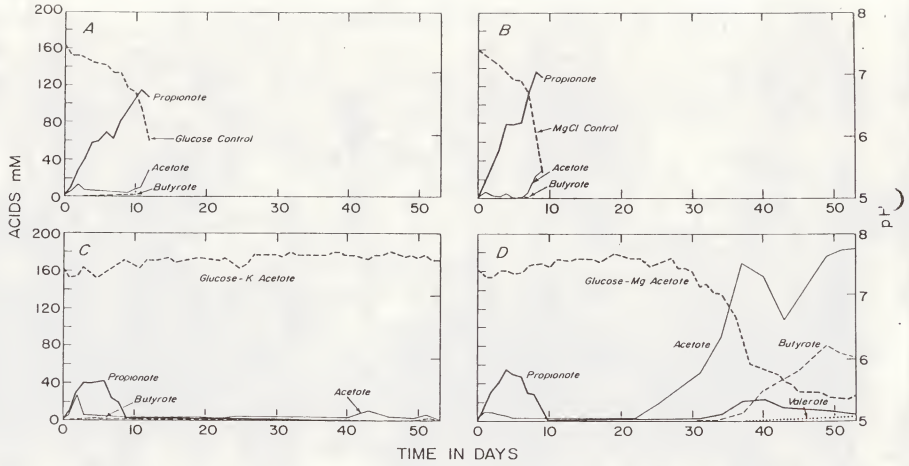
80
100
120
140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Acetate
Butyrate
B
Propionate
MgCl Control
Acetate
Butyrate
C
Glucose - K Acetate
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Butyrate
Acetate
D
Glucose - Mg Acetate
Acetate
Butyrate
Propionate
Valerate
ACIDS mM
pH
TIME IN DAYS

TITLE: [redacted] ^{13}C NMR to Observing Methane Interactions in Anaerobic Digesters

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ABSTRACT

The utilization and conversion of glucose to volatile acids was monitored in anaerobic digesters. Glucose was converted to lactate and acetate. The lactate was subsequently converted to propionate. The distribution of the labeled carbons in propionate suggested that minor amounts were produced via the randomizing pathway and the major portion of propionate was derived from lactate.

INTRODUCTION

Recent studies have demonstrated that high-resolution nuclear magnetic resonance (NMR) provides a rapid and noninvasive technique for the study of metabolism in suspension of intact cells (2, 3, 4, 8, 9). The study of glucose metabolism in E. coli and yeast were especially interesting because intermediate metabolites were evident (4, 9). In the present study we measured the utilization of and the volatile acids produced from ^{13}C-1-glucose in the complex ecosystem of anaerobic digestion of manure and glucose mixtures.

These experiments indicated that glucose utilization could be monitored and, upon the initial feeding of glucose, utilization was delayed. Subsequent feedings of glucose to digest showed rapid utilization of glucose and the production of acetate, lactate and propionate. The relative amounts of ^{13}C-3-propionate to ^{13}C-2-propionate also indicated that propionate was primarily generated by the acrylate pathway (1, 10) and that the randomizing pathway (5) provided minor amounts of propionate. These results suggested that ^{13}C-NMR could be a useful tool in the study of metabolic processes in anaerobic ecosystems.

MATERIALS AND METHODS

DIGEST

Samples of digest were taken from a 3.2l working volume anaerobic digester which had been maintained in a 37°C water bath on a 16 day retention time with cattle manure containing 5% total solids for over three years. The cattle feed, and manure collection and preparation has been described previously (7). Two ml of digest (pH 7.35) was

added to a 10 mm diameter tube containing 7.2 mg of ^{13}C-1-glucose (20 mM glucose). The tube contents were swept with CO_2 to exclude O_2 and thoroughly mixed to dissolve the glucose. A 4.0 mm coaxial tube, containing D_2O and ^{13}C-dioxane was then inserted into the 10 mm tube. The deuterium NMR signal was used as a lock signal and the ^{13}C resonance of the dioxane provided a reference for the carbon spectra. The coaxial tube also served to seal the 10 mm tube containing digest.

The ^{13}C-labeled glucose was a gift from Dr. Jack Nordin, Department of Biochemistry, University of Massachusettes, and used without further purification. Spectra obtained on solutions of the ^{13}C-1-glucose indicated the sample was pure.

SPECTRA

The ^{13}C-NMR spectra at 62.3 MHZ were obtained in fourier transfer mode on a Bruker WM-250 NMR spectrometer. Temperature of the sample was maintained at 37^{o}C. The spectra were fourier transforms of the accumulation of a number of radiofrequency pulses (41^{o} pulse angle, 1.8 sec repitition time). The number of scans collected for each free induction decay depended on the rate of glucose utilization. Eight K data points were collected for each free induction decay. Chemical shifts of glucose, intermediates and products were assigned relative to the resonance of tetramethylsilane as determined from the external reference dioxane.

IDENTIFICATION OF METABOLITES

The chemical shifts of possible intermediates or products of glucose metabolism were identified by spiking digest with these compounds and observing the chemical shifts from the natural abundance ^{13}C nuclei. The compounds for which spectra were obtained were acetate, propionate,

butyrate, pyruvate, lactate, acrylate, malate, succinate and ethanol. Care was taken to maintain the pH near 7.5, the value normally encountered in our digest samples, ~~because~~ since pH changes cause shifts in the resonances.

GLUCOSE UTILIZATION

Glucose utilization in digesters that had first received glucose was compared to those that had received glucose several times. ^{13}C NMR measurements were obtained on digesters receiving glucose on days one and three of the experiment (day one being the first time the digestor had received glucose). These days were chosen based on previous experiments where glucose utilization was observed using the quantitative enzymatic assay of Peterson and Young (6). In the previous experiment it was observed that the glucose utilization on day one was significantly different than those on subsequent days.

The time intervals used for monitoring glucose metabolism in the ~~spectra~~ digesters were varied depending on the day of the experiment. On day one of the experiment spectra were obtained with 1.5 ~~hour~~ hr accumulations (1680 scans). Whereas, on subsequent days of the experiments glucose spectra were obtained with 10 min accumulations (250 scans).

RESULTS

METABOLITES

The resonances of metabolites are shown in Fig. 1. This spectrum was taken approximately 12 hrs after feeding. The products from glucose fermentation were acetate and propionate. In this spectrum it was evident that the resonance from C-2 of propionate had a lesser intensity than the resonance of C-3 of propionate. In a spectrum of

propionate containing the natural abundance of ^{13}C the CH_3:CH_2 intensities were 1.16:1. The ratio of intensities in the spectrum shown was 5.25:1. Only a trace amount of acetate was evident.

In a similar experiment, but approximately 1.5 hrs after feeding, an additional metabolite, lactate, was evident from the spectrum (Fig. 2). Lactate was an immediate and a major metabolic product of glucose metabolism. As time progressed in this fermentation lactate disappeared, propionate accumulated and acetate, again was nearly absent after 12 hrs.

GLUCOSE UTILIZATION

An example of "stacked" spectra is shown in Fig. 3. This shows the changes in the intensities of the α-glucose and β-glucose with time. Plots of intensities versus time for an initial and a subsequent feeding of glucose are shown in Figs. 4a and b. The rate of glucose utilization greatly increased with subsequent feedings over the rate with an initial feeding of glucose.

It was of interest to compare glucose utilization results from the NMR studies with results obtained by traditional chemical analyses. Glucose utilization following an initial and a subsequent feeding as determined by chemical analyses is depicted in Figs. 4a and b. The glucose utilization profiles from both methods were similar. It was evident that glucose utilization increased with time.

DISCUSSION

From the results depicted in Fig. 1 it was apparent that both acetate and propionate were produced from the fermentation of glucose but propionate was not utilized as readily as acetate. Secondly, since

the majority of the ^{13}C label from ^{13}C-1-glucose was found in the C-3 position of propionate we concluded that propionate was produced primarily via the acrylate pathway (1, 10) rather than the randomizing pathway (5), see Fig. 5. The observation that lactate was produced from glucose early in the fermentation (Fig. 2) but was absent later in the fermentation (Fig. 1) suggested that lactate served as a precursor of propionate. This suggestion is consistent with the conclusion that propionate was primarily produced by the acrylate pathway. More detailed studies are currently being pursued regarding lactate and propionate production and utilization.

The data presented in Figs. 3 and 4 suggested that the utilization of glucose was readily monitored by NMR spectroscopy. It was evident that the microbial populations required an acclimation period for utilization of glucose, since subsequent feedings of glucose produced utilization at much faster rates than with the initial feeding.

ACKNOWLEDGEMENT

This work was supported by grants from the Montana Department of Natural Resources and Conservation; Renewable Alternative Energy Division, Grant No. 403-772 and NSF, Grant No. CHE-7826160 and the Montana Agricultural Experiment Station.

We gratefully acknowledge the technical assistance of Mr. Mark Waddington.

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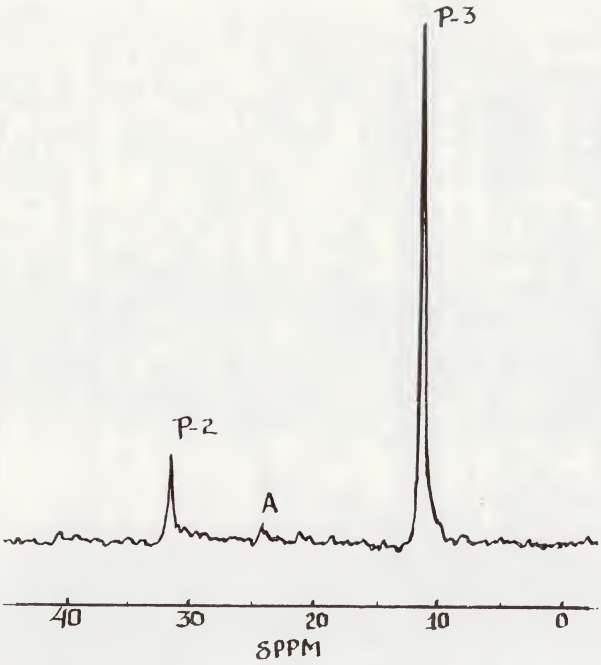
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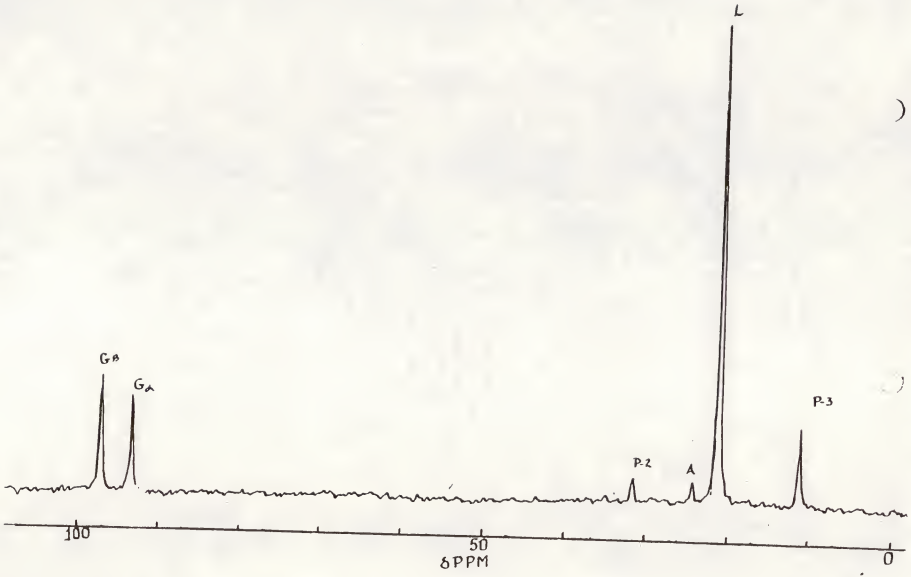
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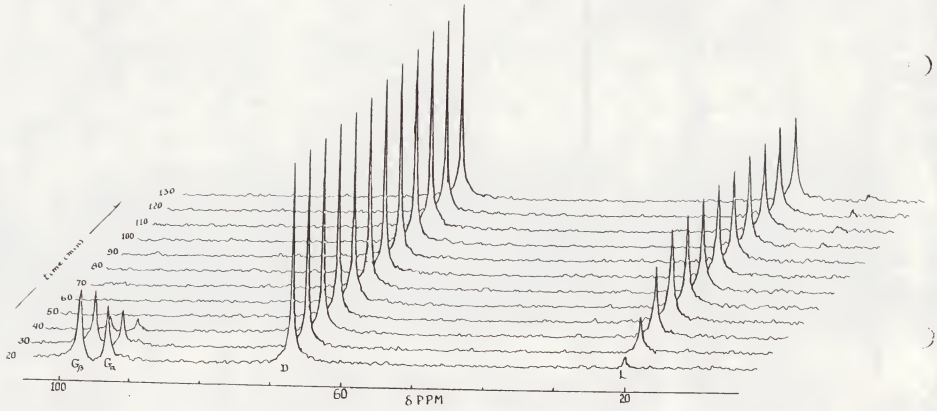
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90
80
70
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50
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G_β
G_α
D
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20
δ PPM

GLUCOSE CONCENTRATION
(mM or relative concentration)
0
5
10
15
20
TIME (hrs)
0
2
4
6
8
10
12
α
β

GLUCOSE CONCENTRATION
(mM or relative concentration)
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15
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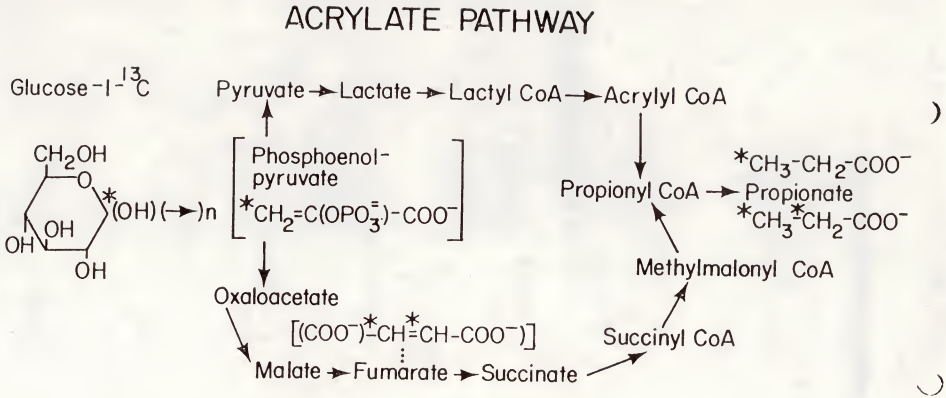
β
α
0
60
120
TIME (min)


ACRYLATE PATHWAY
Glucose-1-^{13}C
Pyruvate
Lactate
Lactyl CoA
Acrylyl CoA
CH_2OH
O
*(OH)(→)n
OH
OH
OH
Phosphoenol-pyruvate
*CH_2=C($OPO_3^=$)-COO^-
Propionyl CoA
*CH_3-CH_2-COO^-
Propionate
*CH_3*CH_2-COO^-
Methylmalonyl CoA
Oxaloacetate
[(COO^-)-*CH=*CH-COO^-)]
Succinyl CoA
Malate
Fumarate
Succinate
RANDOMIZING PATHWAY

[redacted]

FACTORS AFFECTING BIOCONVERSION AND METHANOGENESIS IN ANAEROBIC DIGESTION

Grant No.: RAE 404-801

Time Period: [redacted]

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INTRODUCTION

Anaerobic digesters that are designed for fuel production as a primary purpose rather than solid waste treatment are often stressed by high concentrations of waste materials or short retention times. These practices are becoming more common with efforts to increase the methane yield. The fact that anaerobic digesters become stressed (i.e. their metabolic relationships become unbalanced) makes the process appear to be unstable and therefore unreliable. This perceived instability is a detriment to wide acceptance of anaerobic digestion as a source of renewable energy. The accepted view of the rate limiting process is that the methanogenic population cannot keep pace with the fermentative bacteria under high energy loads. The fermentative bacteria have the capability of producing molecular hydrogen (H_2) as their electron disposal system. (see the reaction below). The concentration of H_2 is considered

eq. 1. $NADH + H^+ \rightleftharpoons NAD^+ + H_2$, $\Delta G^{o'} = +4.3$ kcal/mol

to be critical to the flow of carbon substrate (especially carbohydrates), to acetate or other volatile acids, such as propionate. According to the accepted view the methanogenic bacteria cannot remove H_2 fast enough for the electron disposal of the fermentative bacteria. This results in accumulation of H_2 which then inhibits the formation of H_2 by creating an unfavorable free energy change (ΔG). This resists the production of oxidizing power (NAD^+) needed for continued oxidation of substrates by the fermentative bacteria. The net result then is the fermentative bacteria must seek an alternative electron sink. The production of acids, such as propionate, lactate, succinate and butyrate are known as possible alternatives for fermentative bacteria to produce NAD^+. The production of these acids imposes stability problems on the ecosystem with respect to methane production. The conversion of these acids to acetate which is the most important precursor of methane, is a formidable energy barrier. We think it is important to understand whether the rate limiting process is the H_2 removal by the methanogenic bacteria, shown in eq. 2, or simply the need for NAD^+ by the fermentative bacteria.

eq. 2. $4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O \qquad \Delta G^{o'} = -33$ kcal/mol

The treatment of the digesters to avoid the problem of producing too much propionate, lactate, butyrate, etc. is dependent on knowing which class of bacteria is causing the instability problem.

This study was designed to observe the accumulation profiles of H_2 and volatile acids in anaerobic digesters receiving high energy loads. The major interest was to see if H_2 concentrations could be correlated with the onset of volatile acid accumulations. If H_2 concentration increases to the point where the reaction shown in eq. 1 is inhibited, and if this is the primary cause of the production of the acids, such as propionate, then the accumulation of propionate should commence following the peak hydrogen concentration. Propionate production should also decrease as the H_2 concentration decreases from its maximum value. Further, if the rate of utilization of H_2 by methanogens is the principle cause of H_2 accumulation, then the rate of disappearance of H_2 should be significantly slower than the rate of accumulation.

In previous experiments we have shown the effects of glucose concentration on fermenter stability. Those studies indicated fermenters become unstable and fail when the feed concentration contains glucose at levels which account for 50% or more of the volatile solids. In the present study fermenters received feed containing a glucose concentration which resulted in 60% of the volatile solids (i.e. the glucose concentration was 6% by weight in the feed manure). Thus, these fermenters became highly unstable from the high energy concentration. The daily $[H_2]$ and volatile acid concentrations in the fermenters were observed over a period of seven days on alternate days.

MATERIAL AND METHODS

The daily manure used in these experiments was collected from a holding area within two hours of deposition. The cattle were fed 40% grain mix and 60% hay. The grain mix contained 50% barley, 25% wheat, 10% soybean meal, 10% sugar beet pulp and 5% mustard meal. The manure composition is given in Table I. Total solids (T.S.) and volatile solids (V.S.) were determined by standard methods (1). The fiber content was determined as described by Goering and van Soest (2) and the nitrogen content by the Kjehldahl method. The manure was diluted with tap water to bring it to the desired total solids content. This mixture was subjected to 30 sec. blending in a 3.8 liter Waring blender, then packaged in 1000 ml plastic bags and frozen. This feed was thawed and stored at $4^{o}C$ as needed.

The digest used in these experiments was obtained from a three year old 3.2 l digester that had been maintained on a 16 day retention time (RT) at 37^{o} on manure containing 5% total solids. Two anaerobic digesters were used in this experiment: (1) A 2 l Erlenmeyer flask which served as the primary digester for the experiment, and (2) an experimental fermenter (Figure 1) which periodically received portions of digest from the Erlenmeyer fermenter.

The Erlenmeyer fermenter was maintained on a 16 day RT in a 37^{o} water bath. Feed for the experimental period was 5% total manure solids to which sufficient glucose was added to give a 6% glucose concentration in the manure. Following feeding the digester was swirled for 15 sec. to mix contents. The fermenter was fed only once per day. The experimental digester was given 320 ml portions (400 ml on day -1) from the Erlenmeyer flask one day before experimental samples were drawn. The digester was assembled, swept with CO_2 then digest added with a 50 ml syringe. It was maintained in a 37^{o} water bath. On the day the experimental samples were to be drawn, digest was removed and 5% manure solids and 6% glucose were added by syringe. The digester was then swirled for 15 sec. to mix contents. Two samples were removed at each time point with a 50 ml plastic syringe (B-D) from the exit port at the bottom of the experimental fermenter. These samples were analyzed by the following procedures.

The $[H_2]$ was determined by the procedures of Robinson et al. (3) with the following modifications: (1) 50 ml plastic syringes were substituted for the 50 ml glass syringe, (2) the samples in the syringe were boiled for 15 minutes, and (3) shaking time was doubled, but vigorous shaking was avoided since it caused excessive foaming. The $[H_2]$ in the digest was estimated by the following equation:

$$[H_2] = 1000\ C_i\ V_G\ V_D^{-1}$$

$[H_2]$ = moles/l digest

C_i = $[H_2]$ in GC injection volume (moles/ml)

V_G = gas volume above aqueous NaOH, NaCl column (ml)

V_D = digest sample volume (ml)

The equation for determining $[H_2]$ described by Robinson et al. (3) was not used because an accurate graphical determination of the Bunsen coefficient for digest could not be obtained. Our estimation will therefore be low by some value between 0 and 5%. (For example, our estimate would be 1.3% low using the Bunsen coefficient for water and a 20 ml digest sample in a 50 ml syringe working volume.) GC analysis for H_2 content was performed on duplicate samples. The $[H_2]$ reported is the average of a minimum of four (2 each sample) analyses for each set of duplicates.

Analysis of Volatile Acids

One and one half ml portions of digest samples used in the above procedure were centrifuged. Five hundred µl portions of the obtained supernatants were acidified with 100 µl 25% metaphosphoric acid. The acidified samples were incubated approximately 30 minutes and then analyzed by gas chromatography using a 1.8 m x 2 mm glass column packed with 10% SP-1200, 1% phosphoric acid (Supelco). Column operation was isothermal (115^{o}) with a flow rate of 30 ml/min nitrogen. Detection was accomplished by flame ionization and a Hewlett Packard 3380A integrating recorder.

RESULTS

These experiments were designed to determine the concentration of hydrogen and volatile acids in digest during the hours immediately after feeding large daily quantities of glucose.

Day Zero

The fermenters initially received glucose at time zero (T_0) of day zero. The [H_2] following feeding is shown in Figure 2. The [H_2] increases gradually throughout the sampling period, increasing from .15 µmoles/l to .37 µmoles/l in 8 hours. Similarly, the [acetate] and [propionate] increase gradually throughout this time period. (Fig 2).

Day Two

The fermenters received glucose for the third time at T_0 of day two. The [H_2] at $T_{+½\ hr}$ (.65 µm) was significantly higher than at $T_{-½\ hr}$ (.08 µm). The [H_2] decreased throughout the remaining experimental period but remained above the $T_{-½\ hr}$ concentration. The [acetate] and [propionate] again increased throughout the experimental period. The apparent decrease in [propionate] from $T_{-1/2\ hr}$ to $T_{1/2\ hr}$ is due to dilution by feed manure which has a significantly lower [propionate] than the digest at T_0 of day 2.

Day Four

The digest received glucose for the fifth time on day 4. Again the [H_2] increased immediately after feeding. On this day the [H_2] peaked at approximately 1 µM at $T_{2.5\ hr.}$ Again the [H_2] did not return to the pre-glucose feeding level during the sampling period. The concentrations of acetate and propionate again increased from the time of feeding through $T_{4.5\ hr}$, then decreased slightly from $T_{4.5\ hr}$ to $T_{6\ hr}$.

Day Six

The fermenter received glucose for the seventh time on day 6. The [H_2] increased dramatically after feeding to nearly 3µM. Again the [H_2] decreased with time throughout the remaining portion of the sampling and again did not decrease to the pre-feeding concentration during the sampling period. The acetate concentration through the first 2 1/2 hours

of the experiment, then decreased slightly through $T_{6\ hr}$. Propionate appeared to increase in the first 1/2 hr after feeding then remained constant.

Conclusions

It is evident from the profiles of H_2 and propionate accumulations shown in Figure 2 that the onset of propionate accumulation does not occur after the peak $[H_2]$ is obtained. Thus the inhibition of the production of H_2 and 'oxidizing power' (NAD^+) by $[H_2]$ cannot be the primary cause of propionate accumulation in these digesters under high energy stress. These experiments do not indicate what the primary cause is, however, it is interesting to calculate the ratio of NADH:NAD^+ in an unstressed fermenter, i.e. a fermenter which has not seen a high concentration of glucose. The H_2 concentration found in these digesters initially was approximately 10^{-7}M and the $[H^+]$ was 4×10^{-8}M (pH=7.4). One can approximate the NADH:NAD^+ ratio then by assuming some small negative ΔG value which would allow the reaction shown in equation 1 to proceed. If one assumes ΔG = -0.2 kcal/mol then the NADH:NAD^+ ration would be 5000:1 as calculated by equation 3 below.

eq. 3 $$\Delta G = 4.3 \text{ kcal/mole} + (1.357 \text{ kcal/mol}) \log \left[\frac{(NAD^+) \cdot 10^{-7}_M}{(NADH) \cdot 4 \times 10^{-8}_M} \right]$$

This large ratio suggests the concentration of 'oxidizing power' (NAD^+) in the fermentative bacteria may be limiting.

It is impossible to directly measure the NAD^+ and NADH concentrations in the fermentative bacteria. Indirect measurements of glucose utilization rates and propionate and acetate production rates may give a clue as to the needs of the fermentative bacteria for NAD^+. These studies are currently being pursued.

The rate at which H_2 accumulates and dissipates cannot be accurately assessed by the data obtained in this study. The sampling frequency would have to be greatly increased to estimate these rates. From the data for day 2 the accumulation appears to be faster than dissipation, on days 4 and 6 the rates are approximately equal. Overall the data suggest the rate of production may be slightly greater than utilization.

TABLE I. Composition of Dry Matter of Dairy Cattle Waste

	% of dry matter
V.S.	83
Ether Extract	2.5-2.8
Cellulose	31
Hemicellulose	12
Lignin	12.2
Total nitrogen (crude protein)	2.0(12.5)
Ammonia	0.5
Volatile acids; acetic	0.1
propionic	trace
other	-

BUDGET REPORT

Second Quarter RAE 404-801

	Budgeted	Committed	Balance
Salary & Benefits	$27,025.00	$8,111.23	$18,913.77
Travel	600.00	385.71	214.29
Supplies	2,000.00	422.34	1,577.66
Administration	8,107.00	2,433.37	5,673.63

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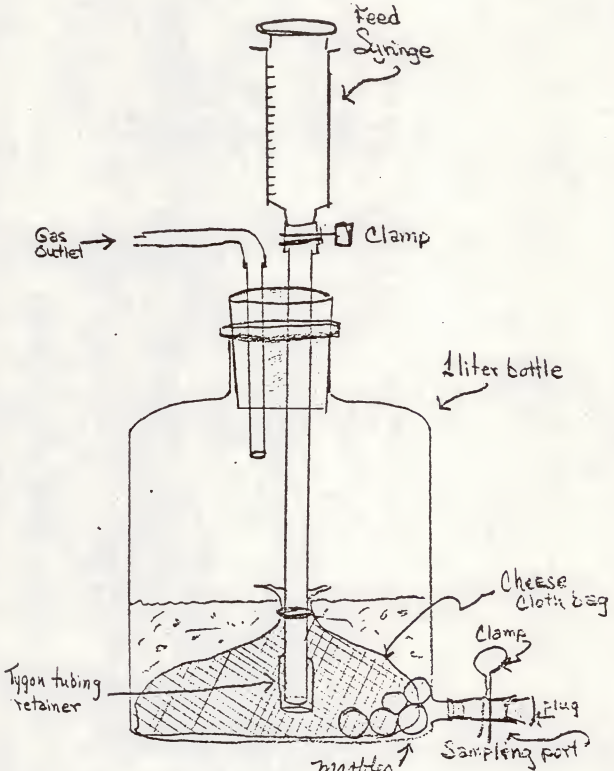
CAPTION FOR FIGURES

Fig. 1 Sampling fermenter. The special features of this apparatus are the cheese cloth bag attached to the feed input tube, the tygon tubing that prevents the cheese cloth from slipping off the feed tube, the marbles, which prevent clogging of the sampling port and the sampling port. The cheese cloth function was to confine the particulates.

Fig. 2 Profile of volatile acid and hydrogen concentrations. Three of four days are depicted. Day zero is described in the text. The abbreviations Pr and Ac designate propionate and acetate respectively. The H_2 reaches a peak within two and half hours after feeding. In general, propionate begins to accumulate prior to that time.

Sampling fermenter for H_2



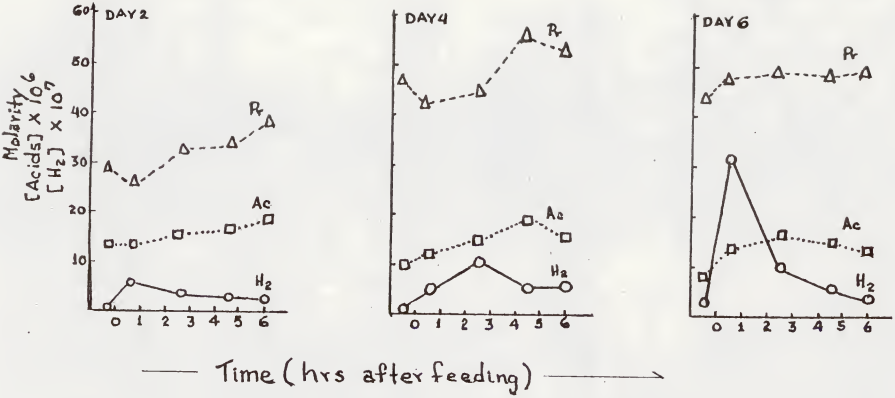


Figure 2: Volatile Acid and H_2 Concentrations

Quarterly Progress Report on

FACTORS AFFECTING BIOCONVERSION AND METHANOGENESIS IN ANAEROBIC DIGESTION

Grant No. RAE 404-801

Time Period: [redacted] 1980
First Quarter

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Submitted to: Montana Department of
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Renewable Alternative Energy Division
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INTRODUCTION

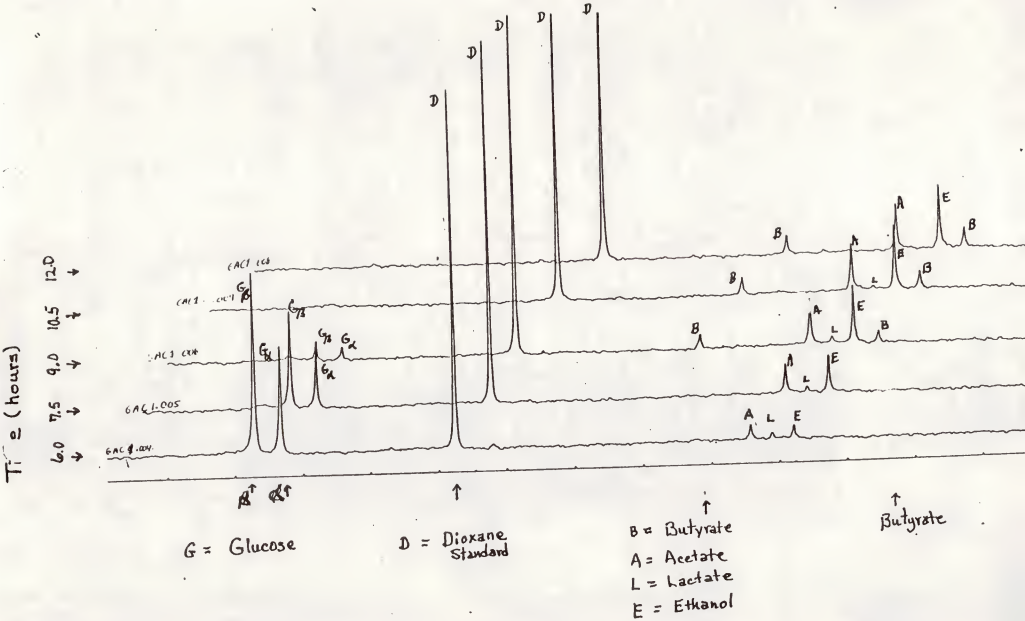
In our studies of the metabolism occurring among the microbial species in anaerobic digestion we sought a new technique for observing changes in metabolic flow under high energy loads. In these first few months we have explored the possibilities of applying ^{13}C-nuclear magnetic resonance spectroscopy (^{13}C-NMR) to the study of anaerobic digesters. The advantages of this technique are:

(1) The bacteria do not have to be removed from the system, killed and the metabolic products extracted from the milieu and then quantitative determinations performed, and

(2) The time requirements for performing an experiment are greatly reduced; from months down to weeks. These advantages we felt were worth the investment of our time and effort.

RESULTS

Some of our data are shown in the accompanying figure. The experiment was performed by adding ^{13}C-1-glucose to 2 ml of anaerobic digest and scanning this mixture over a 12 hour period. The figure shows a time sequence of the results from such an experiment. The peaks that correspond to glucose and the metabolic products are keyed in the figure. It is apparent that glucose is being consumed as noted by the decrease in the height of the glucose peaks with time. Glucose is entirely consumed after 10.5 hours. Also it is evident that products, acetate, lactate, ethanol and butyrate are being formed as noted by their peak height changes with time. Further, one can see that lactate is transient, i.e. formed and then utilized. Dioxane does not participate in the experiment except to serve as a standard for frequency, intensity and quantity. Clearly, these experiments show that ^{13}C-NMR is a useful tool for following the metabolic activities of anaerobic digesters.


Time (hours)
6.0
7.5
9.0
10.5
12.0
G = Glucose
D = Dioxane Standard
B = Butyrate
A = Acetate
L = Lactate
E = Ethanol
Butyrate

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0099-2240/81/090556-04$02.00/0

Vol. 42, No. 3

Application of ^{13}C-Nuclear Magnetic Resonance to the Observation of Metabolic Interactions in Anaerobic Digestors†

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The utilization and conversion of glucose to volatile acids were monitored in anaerobic digestors by ^{13}C-nuclear magnetic resonance. Glucose was converted to lactate and acetate. Lactate was subsequently converted to propionate. The distribution of the labeled carbons in propionate suggested that minor amounts were produced via the randomizing pathway and that the major portion of propionate was derived from lactate.

Recent studies have demonstrated that high-resolution nuclear magnetic resonance (NMR) provides a rapid and noninvasive technique for the study of metabolism in suspensions of intact cells (2–4, 8, 9). The study of glucose metabolism in *Escherichia coli* and yeast was especially interesting because intermediate metabolites were evident (4, 9). In this study, we measured the utilization of and the volatile acids produced from [1-^{13}C]glucose in the complex ecosystem of anaerobic digestion of manure and glucose mixtures.

These experiments indicated that glucose utilization could be monitored and, upon the initial feeding of glucose, utilization was delayed. Subsequent feedings of glucose to digest led to rapid utilization of glucose and the production of acetate, lactate, and propionate. The relative amounts of [3-^{13}C]propionate to [2-^{13}C]propionate also indicated that propionate was primarily generated by the acrylate pathway (1, 10) and that the randomizing pathway (5) provided minor amounts of propionate. These results suggested that ^{13}C-NMR could be a useful tool in the study of metabolic processes in anaerobic ecosystems.

Samples of digest were taken from a 3.2-liter working volume anaerobic digestor which had been maintained over 3 years in a 37°C water bath on a 16-d retention time with cattle manure containing 5% solids. The cattle feed and manure collection and preparation have been described previously (7). A 2-ml sample of digest (pH 7.35) was added to a 10-mm-diameter tube containing 7.2 mg of [1-^{13}C]glucose (20 mM glucose). The tube contents were swept with CO_2 to exclude O_2 and thoroughly mixed to dissolve the glucose. A 4.0-mm coaxial tube, containing D_2O and dioxane, was then inserted into the 10-mm tube. The deuterium NMR signal was used as a lock signal, and the ^{13}C resonance of the dioxane provided a reference for the carbon spectra. The coaxial tube also served to seal the 10-mm tube containing digest.

The ^{13}C-NMR spectra at 62.3 MHz were obtained in fourier transform mode on a Bruker WM-250 NMR spectrometer. The temperature of the sample was maintained at 37°C. The spectra were fourier transforms of the accumulation of a number of radiofrequency pulses (41° pulse angle, 1.8-s repetition time). The number of scans collected for each free induction decay depended on the rate of glucose utilization. A total of 8,000 data points were collected for each free induction decay. Chemical shifts of glucose, intermediates, and products were assigned relative to the resonance of tetramethylsilane as determined from the external reference dioxane.

The chemical shifts of possible intermediates and products of glucose metabolism were identified by spiking digest with these compounds and observing the chemical shifts from the natural abundance of ^{13}C nuclei. The compounds for which spectra were obtained were acetate, propionate, butyrate, pyruvate, lactate, acrylate, malate, succinate, and ethanol. Care was taken to maintain the pH near 7.5, the value normally encountered in our digest samples, since pH changes cause shifts in the resonances.

^{13}C-NMR measurements were obtained on digestors receiving glucose on days 1 and 3 of the experiment (day 1 being the first time the digestor had received glucose). These days were chosen based on previous experiments in which glucose utilization was observed by using the quantitative enzymatic assay of Peterson and Young (6). Since glucose utilization on day 1 was

† Contribution of the Department of Chemistry and the Montana Experiment Station; published as Journal Series no. 1147.

significantly different from that on subsequent days, the time intervals used for monitoring metabolism in the digestors were varied depending on the day of the experiment. On day 1 of the experiment, data were obtained in 1.5-h accumulations (1,680 scans), whereas on subsequent days of the experiments, when glucose utilization was faster, data were obtained in 10-min accumulations (250 scans).

The resonances of metabolites are shown in Fig. 1. This spectrum was taken approximately 12 h after feeding. The products from glucose fermentation were acetate and propionate. In this spectrum, it was evident that the resonance from C-2 of propionate had a lower intensity than the resonance of C-3 of propionate. In a spectrum of propionate containing the natural abundance of ^{13}C, the CH_3:CH_2 intensities were 1.16:1. The ratio of intensities in the spectrum shown was 5.25:1.

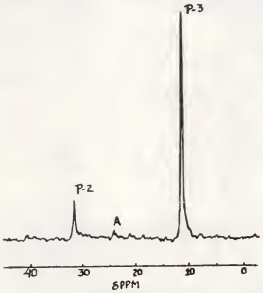

FIG. 1. *NMR spectrum of ^{13}C containing acids 12 h after feeding glucose. A, Methyl group of acetate; P-2, methylene group of propionate; and P-3, methyl group of propionate.*

In a similar experiment, but approximately 1.5 h after feeding, an additional metabolite, lactate, was evident from the spectrum (Fig. 2). Lactate was an immediate and a major metabolic product of glucose metabolism. As time progressed in this fermentation, lactate disappeared, propionate accumulated, and acetate, again, was nearly absent after 12 h.

It was of interest to compare results from NMR observation with traditional chemical analyses. An example of the time sequence of metabolic activity as observed by NMR is shown in Fig. 3. This shows the change in concentration of metabolites with time as reflected by peak intensities. Peak intensities are relative concentrations and not absolute concentrations (3, 4, 9); however, a plot of intensity versus time should correlate with concentration versus time. Glucose utilization profiles for digestors on days 1 and 3 as determined by NMR and the enzymatic assay of Peterson and Young (6) are shown in Fig. 4. The utilization profiles correlate very well.

From the results shown in Fig. 1, it was apparent that both acetate and propionate were produced from the fermentation of glucose but that propionate was not utilized as readily as acetate. Second, since the majority of the ^{13}C

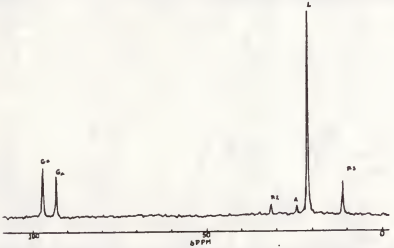

FIG. 2. *NMR spectrum of ^{13}C containing compounds 1.5 h after feeding [1-^{13}C]glucose. A, P-2, and P-3 are as defined in the legend to Fig. 1. G_α and G_β indicate the resonance positions of α and β glucose, respectively, and L designates the resonance of the methyl group of lactate.*

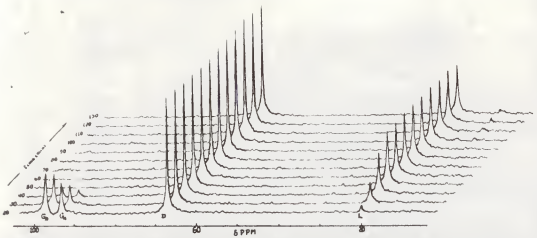

FIG. 3. *Time sequence of NMR spectra which covers 20 through 130 min after feeding. This experiment was performed on digest from a fermentor which had received glucose for the third time. Gα, Gβ, and L are as described in the legend to Fig. 2, and D designates the resonance of the dioxane standard.*

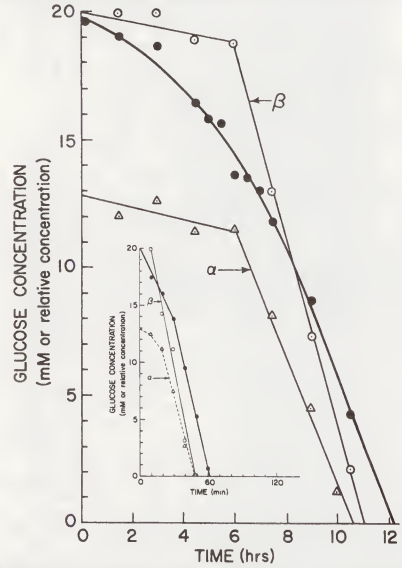

FIG. 4. *Glucose utilization in fermentors receiving glucose the first time. Glucose concentration as determined by chemical analyses (●); α-glucose (Δ) and β-glucose (○) as determined by NMR. The latter are relative concentrations (peak intensities). Inset, glucose utilization in fermentors receiving the third feeding of glucose. The graphic symbols are described for the main figure.*

label from [1-^{13}C]glucose was found in the C-3 position of propionate, we concluded that propionate was produced primarily via the acrylate pathway (1, 10) rather than the randomizing pathway (5). The observation that lactate was produced from glucose early in the fermentation (Fig. 2) but was absent later in the fermentation (Fig. 1) suggested that lactate serves as a precursor of propionate. In general, these observations illustrate the utility of ^{13}C-NMR spectroscopy in the study of metabolism in anaerobic fermentors.

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We gratefully acknowledge the technical assistance of Mark Waddington.

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I. Objectives

1. An evaluation of methane production from different wastes will be made. The wastes to be evaluated will include wheat straw and barley straw for comparative purposes. In addition whey, a by-product of the cheese industry, will be evaluated as a potential source of methane and the digestion process as a possible means of disposal of this waste. These wastes will be evaluated as potential sources of methane and the digester performance compared with the background data gathered from previous studies performed on pure cellulose. The whey studies will be compared with previous studies on protein and sugar additives to digesters. These constituents are both components of whey.

2. A second objective of this proposal is to apply some new analytical methods to assess the metabolic flow of carbon compounds through the anaerobic digestion process. We have recently purchased some ^{13}C-labeled compounds which are representative of important intermediates and precursors in the metabolic processes of fermentation and methanation. These compounds have been tested using ^{13}C-nuclear magnetic resonance spectroscopy. We have been successful at demonstrating the utilization of glucose, the accumulation of the volatile acids, acetate and propionate and the principal pathway for the production of propionate. The success of these initial experiments suggests nuclear magnetic spectroscopy is and excellent tool for direct noninvasive inspection of the metabolic processes in the entire ecosystem of anaerobic digestion. We can easily detect acetate and propionate by this method and thus their production and utilization can be monitored without disrupting the bacterial system. As stated in our previous work, the production and utilization of these acids are critical to the stability of the digesters.

II. Experimental Work

A. Metabolite measurements by ^{13}C-NMR

1. Glucose Utilization

Glucose utilization in high performance digestors was monitored with feeding of ^{13}C-1-glucose and observing the disappearance of glucose via ^{13}C-NMR spectroscopy. As a check on the validity of these determinations glucose utilization was also monitored by traditional chemical analyses. A comparison of these two methods is shown in Fig. 1. The profiles of utilization are comparable and the turnover rate constants obtained were essentially identical within experimental error. The rate constants obtained for the high performance digestors by ^{13}C-NMR and chemical analyses were 1.1 and 1.2 (h^{-1}) respectively. Thus, we concluded that ^{13}C-NMR was a valid tool for observing kinetics of metabolic processes in anaerobic digestion.

Glucose utilization patterns were also monitored via NMR in digestions of glucose and glucose plus acetate. We have previously reported the destabilization of digestions that receive high concentrations of glucose and that destabilization was avoided when acetate was added along with the glucose. These experiments showed that there was no difference in the metabolic flow of glucose carbon between these two types of feedings, Fig. 2. There was a difference in the proportion of acetate and propionate produced when the level of glucose concentration was varied, Fig. 3. In low glucose fermentations (e.g. 6.6mM glucose) no propionate could be detected. The acetate produced was never detectable, thus the acetate was consumed as fast as it was produced. These data suggest that with increased glucose metabolism there was an increased need for alternative electron sinks, such as lactate and subsequently propionate. These data parallel the findings previously reported where nonlabeled glucose was used. We previously suggested that with high concentrations of readily metabolizable substrates, such as glucose, that the buffering capacity of the system could break down and severe destabilization would result by an increase in the acidity. With the addition of acetate the buffering capacity of the digestion was preserved due to production of bicarbonate.

These labeled glucose experiments support this view in that there was a large increase in propionate production which exceeded the utilization capacity. Whereas the acetate production from glucose did not exceed the acetate utilizing capacity. The added acetate may result in enough additional bicarbonate production to offset the increase in propionate.

Measurement of pH and bicarbonate levels in fermentations of high glucose concentrations and glucose plus acetate showed a decline in pH and bicarbonate in glucose only whereas there was an increase in pH and bicarbonate with added acetate. These observations do not directly prove the above suggestions are correct but they are strongly supportive. These findings would be of practicable value in considering digestion of wastes which contained soluble sugars. Wastes containing high concentrations of soluble sugars could arise from utilization of wastes from sugar refineries, fruit cannerys, cheese whey and acid hydrolyzed manure and straw.

2. Propionate catabolism.

We demonstrated the possibility of observing transient metabolic intermediates via NMR spectroscopy by observing the fate of ^{13}C-3-propionate in samples taken from digestors that were acclimated to propionate utilization. The data obtained from such an experiment is shown in Fig. 4. It was evident from this experiment that propionate entered catabolism via a pathway containing lactate as an intermediate. Intermediates of succinate and malate were also evident which suggests a randomizing pathway known for propionate production. Additional experiments indicated that digestions that actively catabolized propionate always produced lactate as an intermediate as well as propionate labeled in the second position. Thus both pathways were active. However, in digestions where propionate was not actively utilized showed evidence of propionate labeled in the second position but no lactate. These observations suggest that propionate was metabolized principally by the lactate pathway. (See Fig. 5).

3. Kinetics on acetate and propionate utilization.

The question we asked concerning reaction rates was which volatile acid, propionate or acetate, is the most recalcitrant. The overall volatilization of organic matter in anaerobic digestion may be limited in the final stages of the digestion. In the mode of operation of once per day feedings of single stage digestors we have shown the principal acids of concern were acetate and propionate. These experiments involved monitoring the rates of utilization of these acids via ^{13}C-NMR spectroscopy. The goal of these experiments was two-fold. One objective was to test this technique for reliability and the second was to obtain relative rates of utilization of acetate and propionate.

In general the rates determined indicated that acetate turnover was faster than propionate turnover. Thus in volatilization of these acids propionate utilization was the rate limiting process in these digestions. The values obtained for first order rate constants were variable. This probably reflects changes in the population of the microbial species. The values obtained for rate constants for propionate and acetate utilization are listed in Table 1. The larger the rate constant value reflects the faster the rate of utilization.

Table 1. Rate Constants for Acid Utilization.

Substrate[1]	k(h^{-1})
Acetate 6-18 P	0.31
" 10-12 P	0.29
" 10-28 PA	0.19
" 11-2 PA	0.12
Propionate 8-9 P	0.05
" 8-18 P	0.05
" 8-27 P	0.16
" 9-11 P	0.15
" 10-15 PA	0.04
" 11-4 PA	0.05
" Chemical-P	0.03
" Chemical-P	0.10
" Chemical-P	0.04

[1]Concentrations used were in the range of 10-20 mM.

These rates are the highest rates we have seen reported thus these digestions are truly high performance. The rate values obtained from chemical analyses (chemical-P) were comparable with the values obtained from ^{13}C-NMR experiments, e.g. 8-9P, 8-18P, 8-27P and 9-11P. Thus we are confident of the NMR technique.

B. Manure and Other Waste Mixtures

1. A comparison of wheat and barley straws mixed with manure.

Mixtures of manure and wheat straw were fermented for methane production and similar fermentations with barley straw were performed. There were no dramatic differences in production of methane between these straws, thus only a brief description of these results are presented here.

Methane production from manure and wheat straw was not much greater than from an equivalent amount of manure solids only. Some fermentations were equivalent to manure but some were 5% higher. Barley straw had less lignin content than wheat straw and those fermentations were on the average 5% better than wheat straw experiments.

In considering the use of straw and the methane yields it would seem that straw used in this manner would not be advisable. The use of straw for feed or to be left in the field would appear to be a better use for Montana. Further, to use straw in digestors requires grinding. This additional energy input further decreases the energy yield.

2. Manure and wheat bran

A fermentation of manure and wheat bran was performed primarily to check the fermentability of bran. A mixture of 5% manure solids plus 3% added bran solids was subjected to fermentation over an 80 day period. The retention time used was 16 days. The digestion was maintained at 37°C with once per day feeding in a single stage digestor. The average daily methane yield was 0.9 vol/vol of digest/day. The average daily methane yield from manure equivalents was 0.68 vol/vol of digest/day. Thus the

bran provided a readily convertible substrate for methane production. The pH remained stable (7.5 to 7.7) and the residual volatile acids were very low suggesting a stable and efficient conversion. In the event of surplus quantities of bran the conversion to methane by anaerobic digestion provides an alternative use of unsalable bran.

3. Manure and whey from cheese production

Here in the Gallatin Valley whey is an unused by-product of cheese production. This whey currently poses a disposal problem for the cheese producer. Whey contains sugar and protein as well as some organic acids. It seemed to us to be an amenable substrate for methane production. After chemical analyses whey was mixed with manure feedstock to provide what we thought should be a good fermentable mixture. This feedstock was utilized in a single stage anaerobic digestor maintained at $37^{o}C$ and on a 16 day retention time with once per day feedings for a period of 160 days. This constitutes 10 turn over periods or 10 retention times. This is sufficient time to establish a steady-state condition. The performance of the digestion was superb.

The pH of the digest remained in the range of 7.5 to 7.8 and after day 80 the volatile acids were 3mM or less. The average daily methane yield was 1.4 volumes per volume of digest.

The composition of whey is shown in the appendix. The whey and manure were mixed to provide a feedstock containing 4.4% solids whey and 5% solids manure for a total solids content of 9.4%. Digestor performance is shown in Appendix B.

III. Conclusions and recommendations

The kinetic analyses performed indicated that rate determinations can be accomplished via NMR spectroscopy. This is a useful tool for research purposes because less time and work are required than rate determinations by the classical methods. These results also indicated that the utilization of the more reduced acid, propionate, was a slower process than the

utilization of acetate. Thus, in the final stage of volatilization of organic matter, the turnover of propionate is the slower process. This emphasizes the importance of the acetogenic class of microorganisms. High performance digestors would require operation which allows enrichment of these populations.

Bran was shown to be an excellent substrate for methane production. This result is attenuated by the fact that bran has other uses of higher economic value and these findings would potentially be beneficial only if there was a surplus of bran in proximity to a methane digestor.

Straws were shown to be convertible substrates but methane production from added straw was not significantly greater than from manure solids alone. The fermentation of straw and manure mixtures would be of value in areas receiving high moisture. However, this is not the case for most of Montana. The straw serves a better purpose left in the field in most of our state.

The good performance of the manure and whey digestion suggests that this could be a very useful disposal mechanism for whey. To be realistic however, this would require the close proximity of a cheese factory and a large dairy.

Both operations would then benefit by an anaerobic digestion of their wastes.

BUDGET REPORT
(10-31-82)

	Budgeted	Expended	Deficit/Balance
Salaries	13,350	14,703.79	-1,353.79
Benefits	1,535	3,282.02	-1,747.02
Supplies	2,997	232.07	+2,764.93
Travel	700	727.00	- 27.00
Administration	4,418	5,395.76	- 977.76
NET			-1,340.64

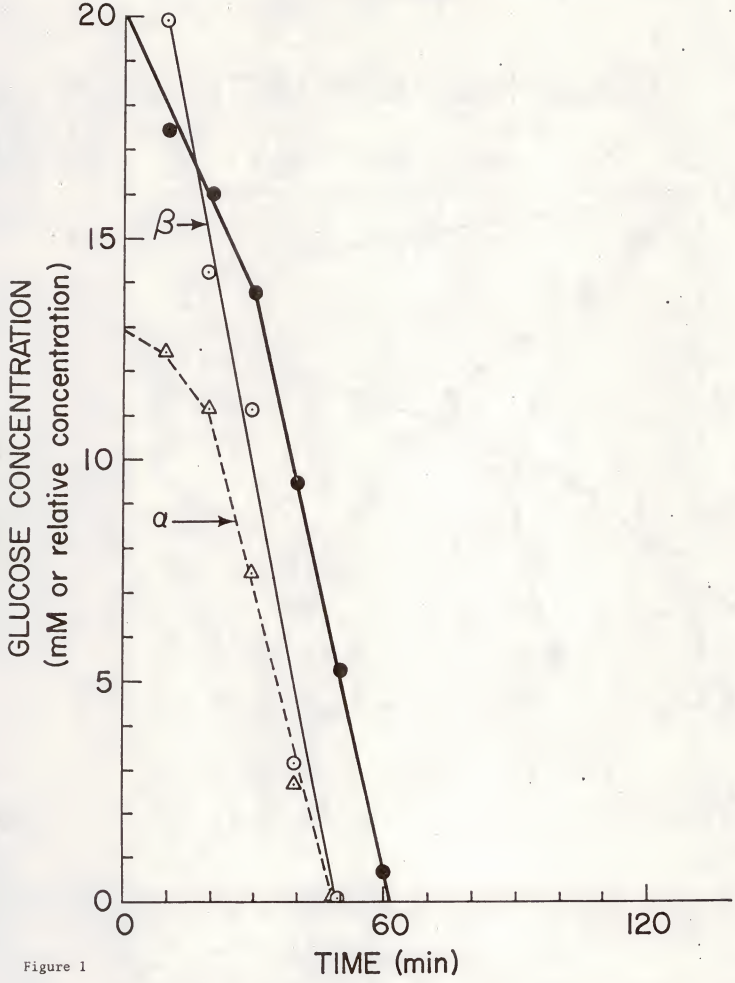
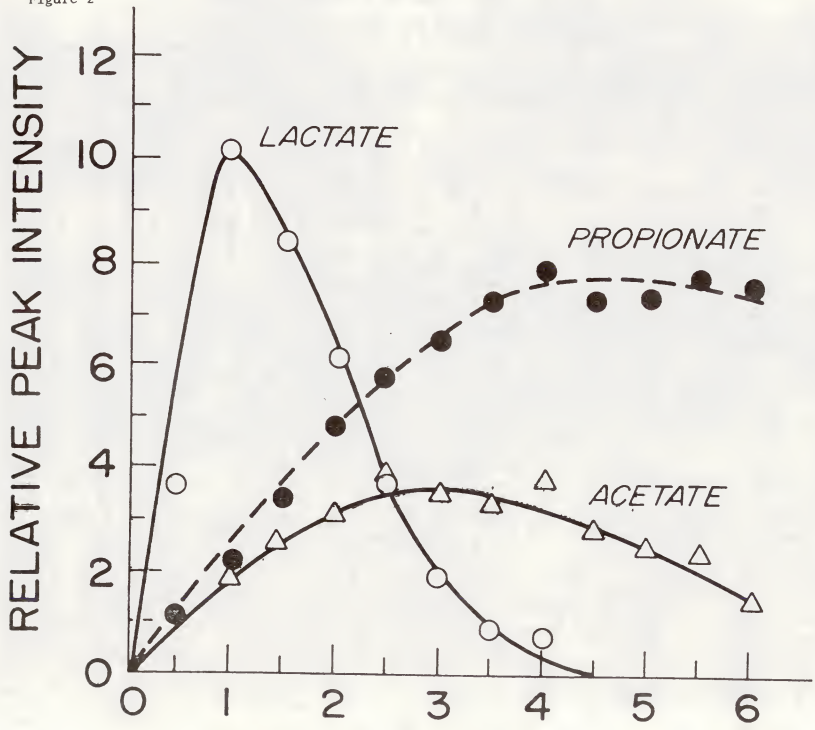

20
15
10
5
0
β
α
GLUCOSE CONCENTRATION
(mM or relative concentration)
0
60
120
TIME (min)

Figure 1

Figure 2


RELATIVE PEAK INTENSITY
12
10
8
6
4
2
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LACTATE
PROPIONATE
ACETATE
0
1
2
3
4
5
6

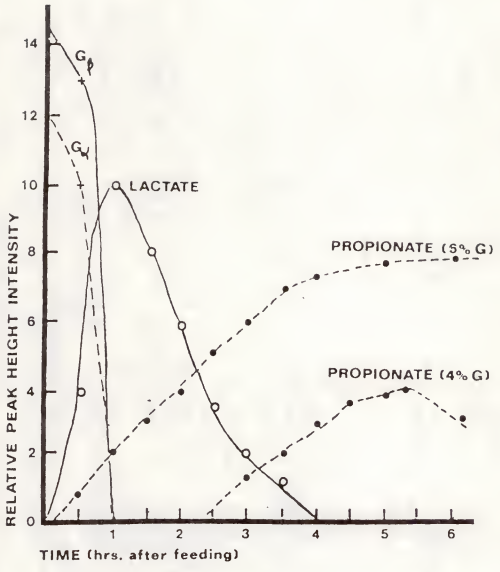


Figure 3. A profile of 2%, 4% and 6% glucose and the resulting metabolites as a function of time as observed by ^{13}C-nmr. The β,γ-glucose (+) and lactate (O) profiles were virtually the same for all three concentrations. The propionate (•) for the 4% and 6% glucose concentrations are as shown.

Figure 4.

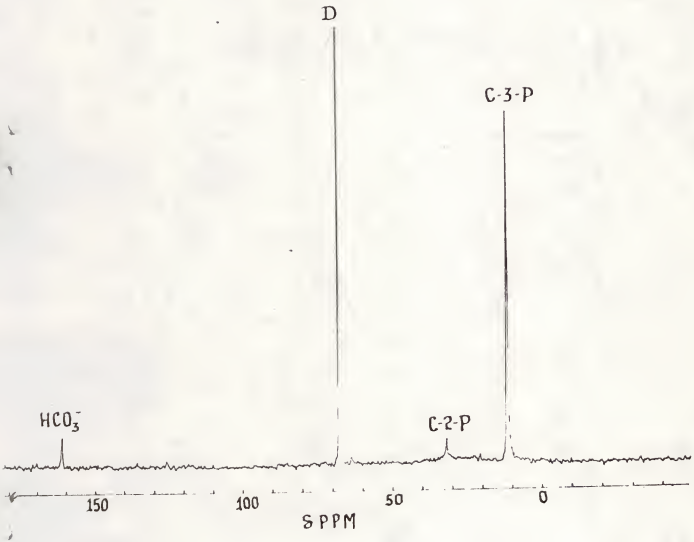


Figure 5.

IV Appendix

A. Composition of whey

Total solids	6.8%
Reducing sugar content	5.2%
(Sugar identified as lactose)	
Protein content (2 mg/ml)	0.2%
Ether extract (lipid)	0.1%

pH = 6.3

Acid Content

Acetic	20.6mM
Propionic	2.8mM
Butyrate	0.3mM
Lactic	6.8mM

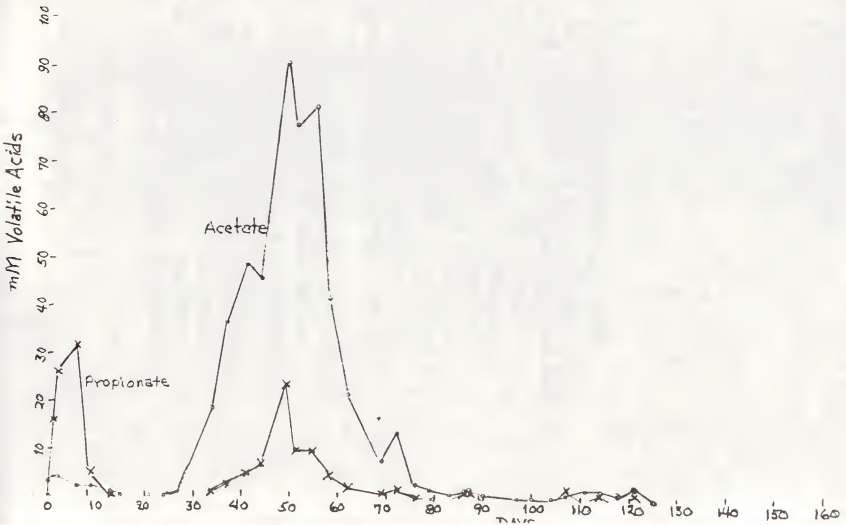
PROGRAM EVALUATION

I would like to compliment the department for supporting fundamental applied research. There is need for fundamental information regarding fermentation processes. The national trend will be toward more basic research and fundamental applied research rather than toward technology demonstrations. The latter have encountered difficulties due to a lack of basic knowledge of the processes. Thus, the department has shown foresight in supporting research projects which aim to elucidate the fundamental processes.

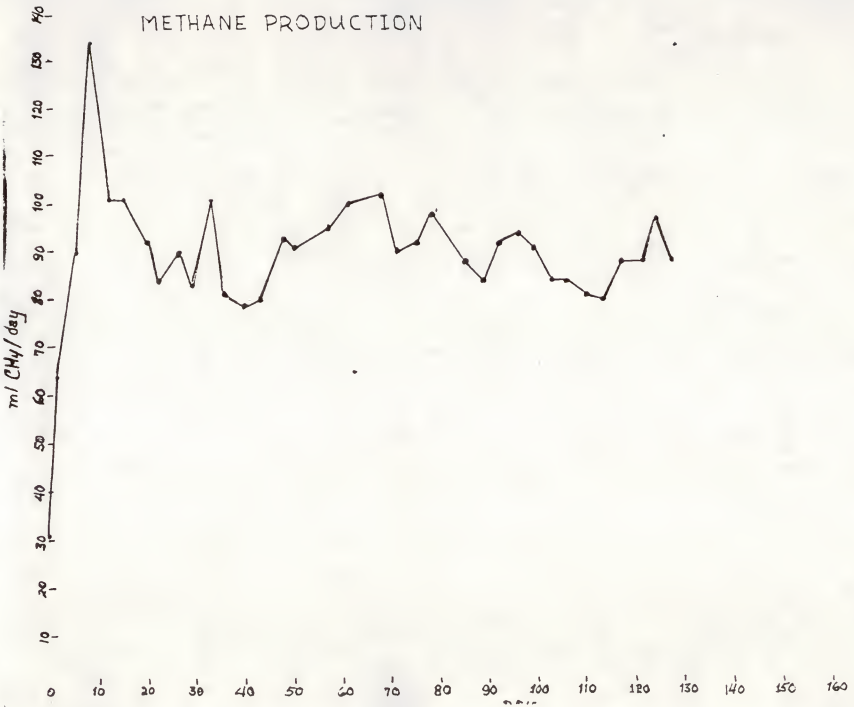
Difficulties have arisen regarding funding. Often the department has approved a project and initiated the funding period long before the funds are available to the project leader. This resulted in less than a year for carrying out the purposed work. On the other hand, when I experienced difficulties in completing proposed work I had no difficulty in obtaining an extension of time.

I am curious as to what the department does with recommendations that researchers and project directors make. I would like to have some information as to the impact of the work I have done.

VOLATILE ACID PROFILE



Appendix B


METHANE PRODUCTION
ml CH4 / day
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160

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